DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date OCT 29 1997

From Acting Director, Division of Programs and Enforcement Policy, Office of Special Nutritionals, HFS-455 5 0 2 '97 NOV 14 P2 :45

Subject 75-Day Premarket Notification for New Dietary Ingredients

To Dockets Management Branch, HFS-305

New Dietary Ingredient:	Huperzine A, an alkaloid compound extracted from the herb Huperzia Serrata.
Firm:	General Nutrition Corporation ("GNC") on its own behlf and on behalf of Marco Hi Tech JV Ltd.
Date Received by FDA:	September 2, 1997
90-Day Date:	December 1, 1997

In accordance with the requirements of section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification for the aforementioned new dietary ingredient should be placed on public display in docket number 95S-0316 after December 1, 1997.

Sincerely yours,

Nicholas Duy for

James Tanner, Ph.D.
Acting Director,
Division of Programs and
Enforcement Policy
Office of Special Nutritionals
Center for Food Safety
and Applied Nutrition

Attachment

95S-0316

RPT 15

GNC LiveWell.™

John P. Troup, Ph.D.
Vice President,
Scientific Affairs

RECEIVED
9/2/97
HFS-450

August 25, 1997

Linda S. Kahl, Ph.D.
Office of Special Nutritionals
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street (HFS-450)
Washington, DC 20204

Dear Dr. Kahl:

Pursuant to Section 8 of the Dietary Supplement Health and Education Act of 1994, General Nutrition Corporation ("GNC"), on its own behalf and on behalf of Marco Hi Tech JV Ltd., wishes to notify the Food and Drug Administration that it will market a new dietary ingredient, Huperzine A, an alkaloid compound extracted from the herb Huperzia Serrata. Accordingly, enclosed please find (2) copies of this notification.

The dietary supplement which contains an extract of Huperzia Serrata, Huperzine A, at a level of fifty (50) micrograms of Huperzine A in a tablet or capsule which will be suggested to be taken one time per day.

Attached please find reports of the safety and other information which establish that this dietary ingredient, when used under the conditions suggested in the labeling of the dietary supplement, is reasonably expected to be safe. These supporting studies include:

(1) Acute oral toxicity of Huperzine A and demonstration of LD50.

(2) A summary description of safety and toxicity studies conducted by international research institutes, a description of the clinical studies conducted in China is also presented.

(3) U.S. Patent describing methods of extraction of the active component (Huperzine A) and review of complete safety/toxicology studies.

5488

General Nutrition Corporation, 300 Sixth Avenue, Pittsburgh, PA 15222
Tel: (412) 338-8844 Fax: (412) 338-8954

(4) Published scientific articles describing the acute and chronic effect of Huperzine A, including sub-population groups.

Very truly yours,

John P. Troup

John P. Troup, Ph.D.
Vice President, Scientific Affairs

JPT/jaj

cc: Reuben Seltzer

Section 3

Huperzine A

US005177082A

United States Patent [19]

Yu et al.

[11] Patent Number: 5,177,082

[45] Date of Patent: Jan. 5, 1993

[54] HUPERZINES AND ANALOGS

[76] Inventors: Chao-mei Yu, Zhejiang Academy of Medicine, Tian Muo Shan Str. Hangzhou; Xi-can Tang; Jia-sen Liu, both of 319 Yue-Yang Road, Shanghai 200031; Yan-yi Han, Tian Muo Shan Str., Hangzhou, all of China

[21] Appl. No.: 599,541

[22] Filed: Oct. 18, 1990

Related U.S. Application Data

[63] Continuation of Ser. No. 305,482, Feb. 2, 1989, abandoned, which is a continuation of Ser. No. 936,005, Nov. 28, 1986, abandoned, which is a continuation-in-part of Ser. No. 795,064, Nov. 5, 1985, abandoned.

[51] Int. Cl.^{5} A61K 31/435; C07D 211/22

[52] U.S. Cl. 514/286; 514/295; 546/63; 546/97

[58] Field of Search 546/63, 97; 514/286, 514/295

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,929,731 5/1990 Kozikowski et al. 546/97

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Canadian Journal of Chemistry, 1989 67 (10) 1538-1540.

Primary Examiner—Robert T. Bond

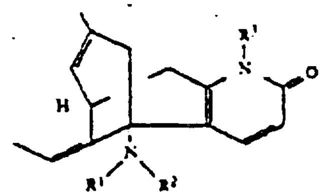
Assistant Examiner—E. C. Ward

Attorney, Agent, or Firm—George M. Gould; William G. Isgro

[57] **ABSTRACT**

The invention relates to compounds of the formulas

I



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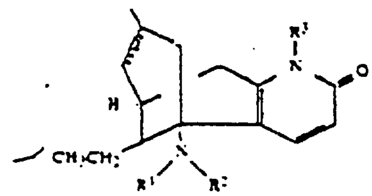

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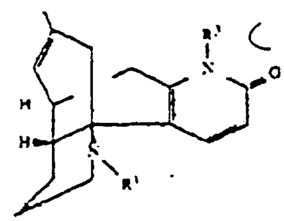


wherein R^1, R^2 and R^3 independently are hydrogen or lower alkyl, and the dotted (. . .) line is an optional double bond, and their pharmaceutically acceptable acid addition salts. The compounds of formulas I, II and III possess marked anticholinesterase activity and are useful as analeptic agents and as agents for the treatment of senile dementia and myasthenia gravis.

10 Claims, No Drawings

HUPERZINES AND ANALOGS

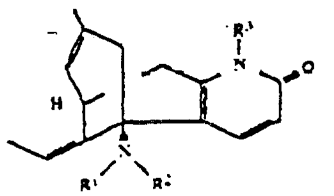
CROSS REFERENCE TO RELATED APPLICATIONS

This is a continuation of application Ser. No. 07/305,882 filed Feb. 2, 1989, now abandoned which is a Rule 60 continuation of Ser. No. 936,005 filed Nov. 28, 1986, now abandoned which is a continuation-in-part application of Ser. No. 06/795,064 filed Nov. 5, 1985, now abandoned.

BRIEF SUMMARY OF THE INVENTION

The invention relates to compounds of the formulas

I



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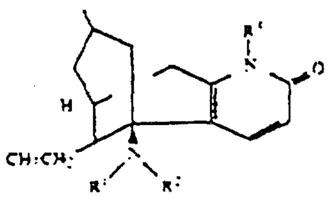

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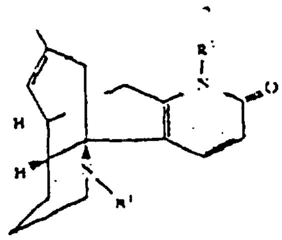
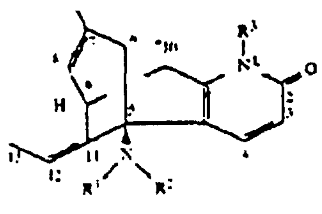


wherein R^1, R^2 and R^3 independently are hydrogen or lower alkyl, and the dotted (. . .) line is an optional double bond, and their pharmaceutically acceptable acid addition salts. The compounds of formula I, II, and III possess marked anticholinesterase activity and are useful as analeptic agents and as agents for the treatment of senile dementia and myasthenia gravis.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to compounds of the formulas

I



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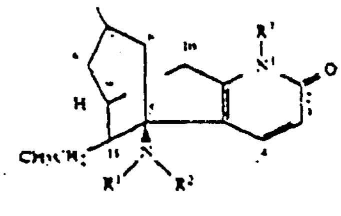

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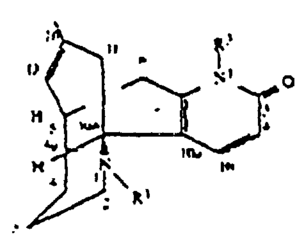


wherein R^1, R^2 and R^3 independently are hydrogen or lower alkyl, and the dotted (. . .) line is an optional double bond, and their pharmaceutically acceptable acid addition salts.

As used herein, the term "lower alkyl" denotes a radical of 1 to 7 carbon atoms, for example, methyl, ethyl, propyl, isopropyl, isobutyl, tertiary butyl, pentyl, heptyl and the like.

The compounds of formulas I, II and III can be prepared as hereinafter described. More particularly, the compounds of formulas I and III, wherein R^1, R^2 and R^3 are hydrogen, which are alkaloids, can be prepared from the naturally occuring plant *Huperzia serrata* by extraction and subsequent chromatographic separation.

Conveniently, the extraction and separation of the desired (5R, 9R, 11E)-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one (Huperzine A) can be effected by known procedures. For instance, a solvent such as an alkanol, for example, ethanol, can be utilized. The extracts obtained can be evaporated and the residue further separated by sequential treatment and extraction as follows. The residue is treated with an inorganic acid, for example, hydrochloric acid. The aqueous phase is neutralized with a base, for example, ammonia or sodium hydroxide, and the total alkaloids extracted by a solvent, for example, chloroform. This sequence can be repeated many times. The final extract can be chromatographed on a silica gel column. Fractions for the chromatography are analyzed by TLC and those with single spots are combined to yield substantially pure Huperzine A. To obtain pure Huperzine A, it can be rechromatographed and recrystallized by known methods, as for example, from a methanol/acetone mixture.

The crude material isolated from later fractions of the chromatography is a minor component which, when rechromatographed on silica gel using, for example, a solvent system of chloroform, acetone and methanol, and recrystallized, for example, from acetone, yields pure (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one (Huperzine B).

The other compounds of formulas I and III can be prepared by alkylation of a compound of formula I or III, wherein R^1, R^2 and R^3 are hydrogen, respectively.

More specifically, the alkylation of a compound of formula I, wherein R^1, R^2 and R^3 are hydrogen, that is, Huperzine A, can be effected utilizing known procedures. For example, if the mono-alkylamino (R^1 is alkyl) derivative is desired, Huperzine A is reacted with an alkyl halide, such as, methyl iodide under standard conditions. If the dialkylamine (R^1 and R^2 are alkyl) derivative is desired, the monoalkylamino derivative is treated further with an alkyl halide, such as, methyl iodide. If the dimethylamine (R^1 and R^2 are alkyl) derivative is desired, it can also be prepared by reacting Huperzine A with a mixture of formic acid and formaldehyde under standard conditions. If the trialkyl (R^1, R^2 and R^3 are alkyl) derivative of Huperzine A is desired, Huperzine A is treated with a dialkylsulfate, such as dimethylsulfate, utilizing standard conditions with heating. In each instance, the desired derivatives can be separated by chromatography and crystallization, or the like.

A compound of formula II can be prepared from the corresponding compound of formula I by selective reduction to either reduce the exocyclic double bond or both the exocyclic and endocyclic double bonds. The exocyclic double bond can be reduced by catalytic hydrogenation utilizing platinum in an alkanol, such as, ethanol, under known conditions. The exocyclic and endocyclic double bonds can be reduced by catalytic hydrogenation utilizing platinum in an organic acid, such as, acetic acid, under known conditions. In each instance, the desired derivatives can be seaprated by chromatography and crystallization, or the like.

The compounds of formula III, wherein R^1 and R^2 are hydrogen, that is, Huperzine B, can be recovered during the separation and recovery of Huperzine A. More specifically, (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one (Huperzine B) can be recovered, as previously described, in the isolation of Huperzine A, initially, as a crude material purified from the later fractions of the chromatography.

The alkylation of a compound of formula III, wherein R^1 and R^3 are hydrogen, that is, Huperzine B, can be effected utilizing known procedures. For example, if the mono-alkylamino(R^1 is alkyl) derivative is desired, Huperzine B is reacted with an alkyl halide, such as, methyl iodide, under standard conditions. If the monomethyl derivative (R^1=methyl) is desired, it can also be prepared by reacting Huperzine B with a mixture of formic acid and formaldehyde under standard conditions. If the dialkyl (R^1 and R^3 are alkyl) derivative of Huperzine B is desired, Huperzine B is treated with a dialkylsulfate, such as, dimethylsulfate, utilizing standard conditions with heating. In each instance, the desired derivative can be separated by chromatography and crystallization, or the like.

The compounds of formulas I, II and III form acid addition salts with inorganic or organic acids. Thus, they form pharmaceutically acceptable acid addition salts with both pharmaceutically acceptable organic and inorganic acids, for example, with hydrohalic acid, such as, hydrochloric acid, hydrobromic acid, hydroiodic acid, other mineral acid salts, such as, sulfuric acid, nitric acid, phosphoric acid, perchloric acid or the like, alkyl, and mono-aryl sulfonic acids, such as, ethanesulfonic acid, toluenesulfonic acid, benzenesulfonic acid,

or the like, other organic acids such as acetic acid, tartaric acid, maleic acid, citric acid, benzoic acid, salicylic acid, ascorbic acid and the like. Non-pharmaceutically acceptable acid addition salts of the compounds of formulas I, II and III can be converted into pharmaceutically acceptable acid addition salts via conventional metathetic reactions whereby the non-pharmaceutically acceptable anion is replaced by a pharmaceutically acceptable anion; or alternatively, by neutralizing the non-pharmaceutically acceptable acid addition salt and then reacting the so-obtained free base with a reagent yielding a pharmaceutically acceptable acid addition salt.

The compounds of formulas I, II and III and their pharmaceutically acceptable acid addition salts exhibit strong cholinesterase inhibiting effects, relatively low toxicity, a large therapeutic index and are superior to physostigmine. Accordingly, the compounds are useful in the treatment of myasthenia gravis and senile dementia. The activity of the compounds of formula I, II and III can be demonstrated in warm-blooded animals, in accordance with known procedures, as hereinafter described:

More specifically, Huperzine A, a representative compound of the invention, is a potent reversible cholinesterase inhibitor which is very selective for specific acetylcholine esterase and it is markedly different from physostigmine. It increased the amplitude of muscle contraction produced by the indirect electrical stimulation of nerves in vitro and using neuromuscular preparations. It also has marked blocking effects against curare. A 1/138 of the LD_{50} dosage of Huperzine A can strengthen the memory functions of normal male rats (Y-maze and brightness discrimination test). The i.p. acute toxicity of Huperzine A is about one-half that of physostigmine in rats and mice. Six months of sub-acute toxicity tests on rats, rabbits and dogs showed that when ninety times the dosage of Huperzine A needed for clinical patients to treat myasthenia gravis and 750 times the equired dosage to treat senile dementia was used, no noticeable pathological changes of internal organs were observed. Mutagenicity test (Ames test) and rat and rabbit teratogenicity tests were all negative for Huperzine A. 3H-labelled Huperzine A was used to carry out pharmacodynamic, distribution and in vivo metabolism research. These studies showed that when 3H-Huperzine A was used the concentration curve matched the open, two compartment model. Its $t_{1/2\alpha}$ = 5.4 minutes and $t_{1/2\beta}$ = 119.5 minutes. There was a certain distribution in the brain which shows that it can pass the blood-brain barrier. There was only a minute quantity of radioactivity in every organ examined after twenty-four hours. Seven days after a single dose 86.1% was eliminated in the urine (84.9% of the excreted drug appearing within twenty-four hours), and 5.5% was eliminated through feces.

Enhancing the Contraction Amplitude of Striated Muscles

1. In Vitro Phrenic Nerve/Diaphragm Preparations of Rats.

After the fast decapitation of a rat, the thoracic cavity was opened and the right diaphragm with attached phrenic nerve was removed. After placing it in a Tyrode's solution (37° C. constant temperature), gassed with 95% oxygen+5% CO_2, electrical stimulation (1-10 V, 0.5 ms, 1 c/10 s) of the phrenic nerve was used to produce muscle contraction. A transducer was used to record the contraction amplitude on a panel recorder. The results are listed in Table 1. When Huperzine A was used in a 0.348 μM concentration, it increased the electrically induced contraction amplitude of muscle by 19%. This action corresponded with the concentration of Huperzine A, showing a very good dose-response relationship. The action of the Huperzine A was slightly weaker than that of physostigmine and neostigmine but much stronger than that of galanthamine.

TABLE 1

The enhancing Effects of Huperzine A on In Vitro Rat Nerve/Diaphragm Muscle Contraction

Drug	Enhancement of Muscle Contraction Amplitude, 50% Concentration (μM)	Strength of Effect
Huperzine A	0.448	1.00
Physostigmine	0.245	1.79
Neostigmine	0.272	1.61
Galanthamine	4.2	0.10
Huperzine B	4.8	0.09

2. Anesthesized Rat and Rabbit Sciatic Nerve/Tibialis Muscle Preparation

Anesthesia was produced in rats by ip injections of 30 mg/kg of pentobarbital and in rabbits by iv injections of 1 g/kg of urethane. Electric stimulation of the periphery of the sciatic nerve (5–10 V, 0.5 ms, 1 c/10 s) caused tibialis contraction which was recorded on smoked paper. The rats or rabbits given iv injections of 30 μg/kg of Huperzine A showed enhancement of the amplitude of the electrically stimulated muscle contraction. Injections of physostigmine, i.v., also enhanced the rabbit's tibialis muscle contraction amplitude but to a lesser degree than that observed for the rats. The potency of Huperzine A in these tests was 1.7 and 4 times that of physostigmine (Table 2). Tubocurarine (0.3 mg/kg iv) completely blocked the electrically induced muscle contraction. After twenty minutes of sustained stimulation, the tibialis muscle contraction amplitude gradually reached the amplitude observed before the injection of tubocurarine. If Huperzine A (40–60 μg/kg i.v.) was given after the i.v. tubocurarine there was marked inhibition of the tubocurarine blockade. Five minutes later, the amplitude of the tibialis muscle contraction was comparable to that seen in the absence of tubocurarine.

TABLE 2

The strengthening Effects of Huperzine A on Whole Neuromuscular Preparations

Drug	Lowest Effective Dosage for Enhancing Muscle Contraction (μg/kg i.v.)					
	Rats	Strength of Effect		Rabbits	Strength of Effect	
Physostigmine	50	1.0		120	1.0	
Galanthamine	500		1.0	500		1.0
Huperzine A	30	1.7	16.6	30	4.0	16.6

ENHANCING THE LEARNING AND MEMORY FUNCTIONS OF RATS

To demonstrate an effect on the learning process a "Y" maze conditioned reflex test was used. Each animal was required to go through 10 successive shock-free runs to be classified as learned. The control animals accepted 11.9±4.9 shocks before achieving the learned state while those receiving 1/50 of the LD_{50} of Huperzine A (0.1 mg/kg, iv) took 6.8±2.8 and those receiving physostigmine (0.08 mg/kg, iv) took 7.9±3.5.

To evaluate the impact on the memory function, preconditioned animals going through 5 shock-free runs were used as learned animals. After 48 hours the drug-free (control) animals required 14.4±8.9 shocks to become learned. With Huperzine A (0.03 mg/kg, ip) only 6.8±7.2 shocks were required while with physostigmine (0.15 mg/kg) 6.4±3.7 shocks were needed to achieve the learned state.

THE IN VIVO DISPOSITION OF ³H-HUPERZINE A

Rats were lightly anesthetized with sodium pentobarbital supplemented with ether and a cannula was placed in the carotid artery. After the animals awoke 1.5, 15 and 30 minutes and 1, 2 and 3 hours after administering iv injections of 375 μCi/kg of ^{3}H-Huperzine A, 0.2 ml of blood was taken from the carotid artery and 0.8 ml of water plus one drop of aqueous ammonia (pH 10) were added to each sample. After adding 5 ml of 1,2 dichloroethane, extraction was effected with the aid of a vortex mixer for three minutes. The aqueous phase was extracted two more times with dichloroethane. After combining the organic phases, the liquid was evaporated to dryness and the residue was placed on silica impregnated filter paper and developed with a mixture of chloroform:acetone:methanol: aqueous ammonia (49:49:1:1) solvent. After chromatographic separation, the 0.5×2 cm band corresponding to the position of non-radioactive Huperzine A was cut out and examined by liquid scintillation techniques. The time curve of ^{3}H-Huperzine A in the blood disclosed an open, two compartment model of distribution. The eliminated phase rate constant and half-life period were separately $\alpha = 0.129$ min^{-1}, $t_{1\alpha} = 5.4$ min, $\beta = 0.0058$ min^{-1}, $t_{1\beta} = 119.5$ min, $K_{21} = 0.0366$, $K_{10} = 0.0204$, $K_{12} = 0.0778$, Vc = 1.04 l/kg, Vd = 3.66 l/kg, the elimination rate was K_{10} and Vc = 21.17 ml/min/kg.

After giving 250 μCi/kg by iv injections of ^{3}H-Huperzine A to the rats, they were sacrificed at different times by bloodletting and the radioactivity contents of the organs were measured. Fifteen minutes after the drug was given, the kidney and liver had the highest contents, the lungs, spleen and heart had less and the fat and brain had the least. Two hours after the drug was given, the radioactivity in the other tissues was markedly lower while that in the brain rose slightly. Twelve hours after giving the drug, the radioactivity in each tissue was close to zero.

Intragastric (ig) injections of ^{3}H Huperzine A (375 μCi/kg) were given 14 hours after the stomachs of the rats were empty and 10 μl of blood was removed from the tip of the tail for measurement of radioactivity. Twenty minutes after the ig injection, the radioactivity in the blood had risen noticeably. It reached a peak in 45–60 minutes after the ig injection and then slowly decreased. Seven hours after the drug was given, the radioactivity in the blood was still relatively high.

After giving a 250 μCi/kg iv injection of ^{3}H-Huperzine A, the urine was collected from 0–6 and 6–24 hours, control urine was collected separately. After chromatographic analysis, a radioactive peak (I) was detected in the R_f 0.65–0.71 area which was identical to that of unaltered ^{3}H-Huperzine A. Another radioactive peak (II) was found in the R_f 0.17–0.21 area and represented a metabolite of the parent compound. The ratio of the two peaks (II:I) gradually increased with the time of urine collection. The II:I ratio was 0.4 after six hours and it was 1.4 in the 6-24 hour period. Thus the drug metabolite was more slowly eliminated into the urine after going through the in vivo process.

Using equilibrium dialysis, it could be shown that the protein binding of ^{3}H-Huperzine A in the plasma of normal mice was 17.2±4.1%.

INHIBITING THE ENZYME ACTIVITY OF CHOLINESTERASE

Red blood cell membranes of rats were used as the source for the true cholinesterase with a substrate concentration of 0.3 mM of S-acetylthiocholine iodide. The source for pseudocholinesterase was 0.1 ml of rat blood serum and the substrate was 0.4 mM S-butyrylthiocholine iodide. The Ellman colorimetric method was used to measure the enzyme activity. The percentage of enzyme activity remaining was plotted against negative logarithm (pI) of the drug concentration and the pI_{50} (the negative logarithm of the gram molecule concentration of the drug required to inhibit the enzyme activity 50%) was derived. Huperzine A inhibited pseudocholinesterase less and true cholinesterase more than physostigmine and neostigmine (Table 3).

A certain quantity of true cholinesterase was mixed with a certain quantity of inhibitor and the enzyme activity was measured at different times after mixing. After the Huperzine A and enzyme were mixed 20 to 30% inhibition was seen very quickly, which did not change over a 6 minute period. The same response was noted for the reversible cholinesterase inhibitors:choline chloride and galanthamine. The irreversible cholinesterase inhibitor DFP, however, yielded increased inhibition with incubation time. Huperzine A yielded inhibition vs time responses similar to those of choline chloride and galanthamine, but different from DFP. Removing the enzyme preparation from a mixture with Huperzine A and then washing restored the activity of the enzyme to 94.4±4.9% of the preincubation value.

The above results show that Huperzine A is a reversible cholinesterase inhibitor.

TABLE 3

Inhibitory Effects of Huperzine A on Cholinesterase

Drug	Inhibition of Cholinesterase (pI$_{50}$) Blood serum	Red Blood Cells
Huperzine A	4.2	7.2
N-dimethyl huperzine A	1.3 × 10^{-3} M ineffective	5.8
N-nonmethyl-huperzine A	1.1 × 10^{-3} M ineffective	5.3
11,12-dihydro-huperzine A	4.9	6.2
tetrahydro-huperzine A	4.3	5.6
N-acetyl huperzine A	1.1 × 10^{-3} M ineffective	<2.5
huperzine B	3.7	6.1
N-methyl huperzine B	3.5	4.1
Physostigmine	5.95	6.65
Neostigmine	5.45	6.65
Galanthamine	4.0	5.7

TOXICITY TESTS

1. Acute Toxicity

A single toxic dose of Huperzine A to mice, rats, rabbits and dogs yielded the typical symptoms of cholinesterase inhibitor poisoning, such as whole body muscle fiber twitching, drooling, tears, increase bronchial secretions and incontinence of feces and urine. The acute toxicity of physostigmine was 1.25 and 1.08 times greater than Huperzine A in mice and rats and both were greater than that of galanthamine. The iv route was most toxic and the ig route least toxic for Huperzine A in rats and mice (Table 4). Ten conscious rabbits were separately given im or iv injections of 0.5-2 mg/kg of Huperzine A and were observed to display the above mentioned toxic side effects for 3-4 hours. One of the two rabbits given iv injections of 2 mg/kg of Huperzine A died. This dosage was 66 times the effective dosage for enhancing muscle contraction. Six dogs anesthesized with chloralose were separately given 0.306 and 1 mg/kg iv injections of Huperzine A with no noticeable effects on the carotid artery blood pressure and EKG.

2. Subacute Toxicity

Rats: 20 male rats were separated into two groups. The first group was given 0.3 mg/kg ip injections of Huperzine A for 51 days while the second group (controls) received the same schedule of distilled water. The routine blood tests (the percent hemoglobin, numbers of red and white cells as well as platelets), zinc turbidity, creatinine and urea nitrogen were all normal. In another test 70 rats were divided into 6 groups. One was given ip injections of 0.5 mg/kg (10 rats) another 1.5 mg/kg (10 rats) of Huperzine A and a third group (10 rats) received only distilled water each day for 90 days. The remaining groups were given ig injections of 1.5 mg/kg (15 rats), 3 mg/kg (15 rats) of Huperzine A each day for 180 days.

A small number of those in groups given large dosages died within 30-150 days while those which survived were sacrificed for examination. The glutamic-pyruvic transaminase values of individual rats from the group given ip and ig injections of 1.5 mg/kg dosages were slightly higher than those of the control group. However, no noticeable effects on the routine blood tests, blood sugar, urea nitrogen, zinc turbidity, musk oxaphenol turbidity and ECG were detected. Microscopic examination of various organ sections showed that the heart muscle had dot-shaped and slice-shaped inflamed areas accompanied by myocardial cell denaturation atrophy. Cerebral spongiocyte growth and myophagia was noted and a small number of rats had sperm cell growth inhibition and interstitial growth. No abnormalities were observed in the other organs.

Rabbits and dogs: there were 20 rabbits divided into four groups. They were separately given im injections of 0.6 mg/kg of Huperzine A for 180 days and iv injections of 0.3 mg/kg and 0.6 mg/kg of Huperzine A for 90 days. The control group was given im injections of distilled water. Three of the rabbits given im injections of 0.6 mg/kg of Huperzine A died between 66-136 days of taking the drug, but no toxic reactions were observed before they died. Ten dogs were separately given im injections of 0.3 and 0.6 mg/kg (3 dogs each) of Huperzine A and distilled water (4 dogs) for the control group for 180 days. No abnormalities were observed in the group given small dosages, but at the 0.6 mg/kg dose there was noticeable whole body muscle fiber twitching. The symptoms gradually decreased and disappeared following the length of the time the drug was given. The ECG showed no drug induced abnormalities. When the time arrived, the rabbits and dogs were dissected. The routine blood tests glutamic-pyruvic transaminase, zinc turbidity, urea nitrogen and creatinine were all normal. Each organ section was observed microscopically and a small number of rabbits in the group given the drug had myocardial cell denaturization atrophy and interstitial growth focus in their hearts. The hearts of the dogs had light fat infiltration. The cerebral cortex of each dosage group of rabbits and dogs had cerebral spongiocyte growth and myophagia, but the nerve pronuclei did not show any retrogression. This shows that when a relatively large dosage of Huperzine A was used for a longer period of time, this could affect the nervous systems of the heart and brain. The stimulation of the latter was even more outstanding.

TABLE 4

Acute Toxicity of Huperzine A on Mice and Rats

Drug	Animal	Means Drug Was Given	LD_{50} (95% Fiducial Limits mg/kg)	Toxic Strength
Huperzine A	Mice	sc	3.0 (2.2–4.1)	1.00
	"	ig	5.2 (3.8–7.2)	
	"	iv	0.63 (0.58–0.68)	
	"	ip	1.8 (1.6–2.2)	
Physostigmine	"	ip	0.8 (0.7–1.0)	2.25
Galanthamine	"	ip	13.4 (11.3–16.0)	0.13
Huperzine A	Rats	ig	25.9 (23.2–29.0)	1.00
	"	iv	2.5 (2.3–2.7)	
	"	ip	5.0 (4.2–5.9)	
Physostigmine	"	ip	2.4 (2.3–2.6)	2.08
Galanthamine	"	ip	22.4 (20.3–25.9)	1.32

3. Mutation Tests

The Ames method as well as the two types of bacteria TA_{98} and TA_{100} which carry different mutation R factors were used to evaluate mutagenicity when combined with a metabolic activation system (S_9 mixed liquid). Four dosages of Huperzine A, 1, 10, 100 and 1,000 µg/container, were used and compared with a cyclophosphamide and a mutation group. Each dosage was run in triplicate with TA_{98} or TA_{100} and an automatic colony counter was used to count the number of reverse mutation colonies. The test results showed that there were no noticeable differences between Huperzine A and the spontaneous reverse mutation colony number. Further, the colony number of the positive control drug (cyclophosphamide) was greater than that of the spontaneous reverse mutation group (Table 5).

TABLE 5

Mutation Tests of Huperzine A ($\bar{X} \pm SD$)

Drug	Dosage (µg/container)	$TA_{98} - S_9$	$TA_{100} - S_9$
Mutation	—	18 ± 13	150 ± [illegible]
Huperzine A	1	24 ± 9*	117 ± 22*
	10	30 ± 17*	83 ± 24*
	100	33 ± 6*	101 ± 25*
	1000	23 ± 7*	91 ± 25*
Cyclophosphamide	1500		566 ± 10**

Compared with spontaneous reverse mutation, *p > 0.05, **p < 0.05

4. Teratological Tests

6–15 days after mice became pregnant they were given ip injections of Huperzine A and 7–18 days after rabbits became pregnant they were given im injections of Huperzine A. The results showed that the number of embryo absorptions and stillborn fetuses for the mice given ip injection of 0.19–0.38 mg/kg of Huperzine A was markedly greater than those of the control group ($P<0.01$). The results of a single ip injection of 0.38 mg/kg of Huperzine A on the tenth day of pregnancy were similar to that obtained when the drug was given many times (Table 6). Neither of the two methods of giving the drug resulted in abnormal embryos seen with the positive drug control of cod-liver oil (each gram contained 50,000 international units of Vitamin A and 5,000 international units of Vitamin D). The latter produced various types of externally observed deformities: short tails (44/97), short and no tails (18/97), back legs reversed (13/97), open eyes (7/97), exposed brains and spina bifida (1/97), sunken noses (1/97) and cleft palates (39/40). The number of stillborn fetuses among the rabbits given im injections of 0.08 mg/kg of Huperzine A was noticeably higher ($P<0.05$) than that of the control group. The other dosage groups both higher and lower had values close to those of the control group ($P<0.05$) (Table 6). No external, internal organ or skeletal deformities were observed for any of the dosages.

TABLE 6

The Effects of Huperzine - A on the Fetus of Pregnant Mice and Rabbits

(1)	(2)	(3) (mg/kg)		(4)	(5)	(6) (7)	(8) (g)	(9) (cm)	(10)	(11)
	(14)		ip	6-15	16	10 ± 2	1.04 ± 0.13	2.1 ± 0.2	0.13 ± 0.34	0.06 ± 0.25
(12)	"A"	0.019	ip	"	4	8 ± 3	1.24 ± 0.22	2.2 ± 0.1	1.0 ± 1.7	0.22 ± 0.44
	"	0.038	ip	"	12	8 ± 2	1.2 ± 0.2	2.2 ± 0.3	0.5 ± 0.8	0.22 ± 0.44
	"	0.06	ip	"	9	9 ± 2	1.2 ± 0.1	2.3 ± 0.1	0	0.17 ± 0.39
	"	0.19	ip	"	15	9 ± 4	1.0 ± 0.2	2.1 ± 0.2	0.73 ± 1.7***	0.13 ± 0.35
	"	0.38	ip	"	10	6 ± 4	1.1 ± 0.3	2.0 ± 0.3	2.3 ± 2.4***	0.8 ± 1.1***
	"	0.39	ip	10	8	8 ± 4	0.95 ± 0.18	1.9 ± 0.1	0.75 ± 1.0***	0.9 ± 1.1***
	(15) AD	0.3	ml/ig	8-10	17	6 ± 3	1.2 ± 0.1	2.2 ± 0.1	3.3 ± 4.5	0
	(16)	0.5	ml im	7-18	4	9.2 ± 0.3	43.9 ± 1.2	8.9 ± 0.9	0	0.25 ± 0.5
(13)	"A"	0.2	im	"	2	9.3 ± 0.6	40.2 ± 2.4	8.9 ± 0.1	0	0.7 ± 0.6
	"	0.08	im	"	6	6.7 ± 2.5	41.2 ± 3.9	9.1 ± 0.3	0	1.2 ± 1.2**
	"	0.04	im	"	3	7.3 ± 3.8	48.0 ± 3.2	9.0 ± 0.1	0	0.7 ± 1.2
	"	0.02	im	"	2	7.0 ± 1.4	42.3 ± 10.4	8.8 ± 0.4	0	0

**p < 0.05

***p < 0.01

A-Huperzine - A

Key: (1) Animal, (2) Drug, (3) Dosage, (4) First day drug given after pregnancy, (5) Number of pregnant animals, (6) Fetus of mice (rabbits), (7) Number of fetuses, (8) Body weight, (9) body length, (10) Number absorbed, (11) number of stillbirths, (12) Mice, (13) Rabbits, (14) Distilled water, (15) Vitamin A and D [illegible], (16) Distilled water

OBSERVATIONS ON THE CLINICAL CURATIVE EFFECTS OF HUPERZINE A ON 128 CASES WITH MYASTHENIA GRAVIS

In order to further verify Huperzine A's clinical curative effects and observe its side effects, trials were undertaken to observe the similarities and differences between Huperzine A and neostigmine. 128 patients with correctly diagnosed myasthenia gravis were used in the trial. 69 of these patients took neostigmine as a control group and 59 patients used Huperzine A exclusively. The conditions of the clinical use of Huperzine A for these 128 cases are set out hereafter.

I. METHODOLOGY

Patients affected with myasthenia gravis (MG) with typical clinical symptoms which improved after using neostigmine were the subjects for testing and verification. Intramuscular injections of Huperzine A were given each day and the curative effects and side effects were observed after the injections. It was generally used for at least ten days and each dosage was 0.4–0.5 mg. Neostigmine and Huperzine A were used to carry out double blind cross-over control trials wherein 0.4 mg of Huperzine A was injected for five days and 0.5 mg of neostigmine was injected for five days with alternating use of the drugs in the control group. The injections were all given in the morning and on the morning prior to the injections anticholinesterase drugs were discontinued. Neither the patients nor the doctors knew which drug was being injected. Later, the symptoms, the duration of the improvements, if any, which were obtained by the drugs and the side effects were recorded. Based on these factors, the relative merits of the two drugs were established.

II. THE SYMPTOM APPRAISAL STANDARDS

(+) (+ +) and (+ + +) was used as the standard for the seriousness of the symptoms. (+ + +) was the most serious.

1. Prolapse of eyelids: the tear width of the eye after use of the drug was measured. If there was an increase of 0.2 cm above that before use of the drug, then the effect was "+", if the increase was 0.4 cm then the effect was "+ +" and if the increase was 0.6 cm then the effect was "+ + +".

2. Impairment to eyeball activity: when the eyeball was basically fixed and immovable then it was "+ + +", those who had reoccuring major complaints and basically normal activities were "+" and those in an intermediate state were "+ +".

3. Difficulties in swallowing: when swallowing was still possible but there was a feeling of difficulty or there was slowing of the speed of the intake of food then the patient was treated as "+"; when the patient could swallow but it was very slow then the patient was "+ +"; when the patient was basically unable to swallow the rating was "+ + +".

4. Systemic myasthenia: patients who were able to walk but felt very exhausted were "+"; patients who were able to stand up and walk with difficulty a short distance in the ward or corridor were "+ +"; and patients who could not get out of bed were "+ + +".

III. CLINICAL DATA

1. Age, Sex, Type and the Course of the Disease

Based on the clinical symptoms, those patients who only had their extra-ocular muscles affected were of the eye muscle type, 83 cases (64.85%) in this group. Those who mainly had tired muscles when swallowing were of the medulla oblongate type, 10 cases (7.81%) in this group. Those who had tired muscles in the four limbs were of the systemic type, 35 cases (27.34%) in this group. The shortest course of the disease was 3 days, the longest 23 years and the average was about 33 months.

62 of the cases in this group were male and 66 were female. The youngest male patient was one year old and the oldest was 80. The youngest female patient was 3 years old and the oldest was 74. The average male and female age was 27.39 years of age.

2. Results After the Use of Huperzine A

(1) Aside from one of the 128 cases, all of the other patients had reactions to the Huperzine A as regards the physical symptom initial improvement time and the optimal curative effect time. The shortest physical symptom initial improvement time was 10 minutes after injection. An individual case had the longest of 3.7 hours before there were effects. The average was 21.92±19.56 minutes. 108 of the cases (85.03%) had effects within 15–30 minutes.

As regards the occurence of the time maximal effect among 127 of the cases for which the drug was effective, the shortest was 18 minutes, the longest was 240 minutes and the average was 50.34±25.65 minutes. 65 cases (51.18%) had the optimal curative effect occur within 45–60 minutes after using the drug. See Table 7.

TABLE 7

The physical symptom improvement initial times and optimal curative effect times of 127 cases with MG.

Types/Time	Shortest (Minutes)	Longest (Minutes)	Average (Minutes) X ± SD	15–30 Minutes No. of Cases	15–30 Minutes %	45–60 Minutes No. of Cases	45–60 Minutes %
initial effect time	10	222	21.92 ± 19.56	108	85.03		
Maximal effect time	18	240	50.34 ± 25.65			65	51.18

2. The sustaining time of the effects of Huperzine A: the shortest sustaining time of the effect of Huperzine A was 0.66 hours and this was a patient on the eye muscle type. The longest was 24 hours and this was observed in the systemic type as well as the eye muscle type. The average action time was 5.94±4.92 hours. The action time of 44 cases (34.64%) reached 4–6 hours while the action time of 40 cases (31.64%) exceeded 6 hours. The shortest time among these 40 cases was 6 hours, and the longest was twenty-four (24) hours, average was 10.42±5.80 hours.

3. Effects

Aside from one case, the drug was effective for the other 127 cases (99.21%). Among these, 71 cases (55.46%) had marked effects and it was effective for 56 cases (43.75%).

4. Laboratory Examinations

Albumin, hemochrome, blood platelet, routine urine, liver function and EKG examinations on some of the 128 cases given Huperzine A before and after they took the drug were carried out and none of them showed any noticeable differences in albumin, blood patelets and routine urine tests before and after being injected. The white blood cells noticeably decreased after the injections and this occurred in only 2 cases (2.4%). 2 cases had abnormal liver functions before the injections and both of these cases had normal liver functions after the injections. However, there were also 2 cases (2.2%)

which had normal liver functions before the injections but the SGPT was abnormal after the injections.

The EKGs of 96 patients before the injections of Huperzine A were recorded and among these 11 cases (11.45%) were abnormal. The EKGs of 72 patients after the injections of Huperzine A were recorded and among these 11 cases (15.27%) were abnormal. 9 of these 11 were among the original abnormal group and only 2 cases (2.7%) were normal before the injections (see Table 8).

TABLE 8

EKG changes before and after the injections.

Sex	Age	EKG Manifestations Before the Injections	Manifestations After the Injections
Female	24	Right bundle-branch Block	Same
Female	50	Incomplete left bundle branch block	Same
Male	22	Pre-excitation Syndrome	Same
Male	31	High voltage	Same
Male	56	Ventricular flutter	Abnormal
Male	34	Frequent early ventricular plus	Same
Female	74	Atrial trembling	Same
Male	10	Frequent early ventricular pulse	Same
Male	60	The left ventricle had high voltage	Same
Male	16	Slight abnormality	Normal
Female	33	Slight abnormality	No follow up visit
Female	24	Normal	ST section change
Female	33	Normal	Light T wave change

5. Comparison of the Effects of Huperzine A and Neostigmine

(1) Comparison of the maintained times of the effects. Control tests were carried out on 69 cases. The action time Huperzine A was longer than that of neostigmine for 58 cases (84.05%) of the action time of neostigmine was longer than that of Huperzine A in 6 cases (8.69%). The action times of the two drugs were close in 5 cases (7.26%). After statistical analyses, there were very significant differences between the two ($X^2=78.52$, $p<0.0001$).

Among the 58 cases wherein the action time of the Huperzine A was longer than that of neostigmine, the shortest difference was 0.05 hours, the longest was 20 hours and the average was 2.90 ± 3.64 hours (see Table 9).

TABLE 9

Specific conditions of 58 cases when the action time of Huperzine-A was longer than that of neostigmine

Time	Difference within 2 Hours	Difference 2-4 Hours	Difference 4-6 Hours	Difference over 6 hours	Average Difference $X \pm SD$ (hours)
58 cases	29	18	4	7	2.90 ± 3.64
%	50	31.03	6.89	12.08	

Among the 6 cases wherein the action time of the Huperzine A was less than that of the neostigmine, the shortest was 0.3 hours and the longest was 6 hours. Four of these cases were within one hour while the other two were 1.6 and 6 hours.

(2) Comparison of the action strengths: the injected dosage of Huperzine A was 0.4 mg whereas 0.5 mg of neostigmine was used. Given these dosages, the action of the former was stronger than that of the latter in 16 of the cases. The action strength of the former was lower than that of the latter in 7 cases. There were basically no differences between the two in 46 of the cases and it can therefore be said that under these dosages the action strengths of both are not very different.

(3) Comparison of the side effects: among control patients, 34 cases had side effects from the neostigmine (49.27%) whereas 45 cases (65.21%) had side effects from the injections of Huperzine A. Statistical analyses showed that there were no significant differences ($X^2=3.58$, $P>0.05$).

Among the more frequently oocuring side effects were perspiring, nausea and blurred vision. These three revealed marked differences statistically between the two drugs (these were separately nausea $X^2=15$, $P<0.001$; perspiring $X^2=5.5$, $P<0.01$; blurred vision $X^2=12.96$, $P<0.001$). There were no marked differences in the occurrence rates of other side effects. Therefore, neostigmine more noticeably than Hyperzine A caused perspiring and blurred vision but Huperzine A was more apt to cause nausea than was neostigmine. If one compares the use of Huperzine A for 128 patients and the use of neostigmine for 69 cases, only in the area of nausea was the percentage of its occurrence greater than that of neostigmine. There was significant statistical difference ($X^2=4.99$, $P<0.05$). The Huperzine A had lower side effects for each of the other items than neostigmine including muscle bundle quivering, dizziness, perspiring and blurred vision. Statistical analysis showed significant difference. ($x^2=4.18$, $P<0.05$, $x^2=36.25$, $P<0.001$, $X^2=25.23$, $P<0.001$, $X^2=46.52$, $P<0.0001$ respectively.) See Table 10. Both the statistics and processing showed noticeable differences and we can thus basically come to the conclusion the Huperzine A is superior to neostig-mine. This is especially true as regards the action time length of Huperzine A which is its outstanding feature. This is actually the major drawback in the clinical use of neostigmine.

(4) Comparison between Huperzine A and neostigmine: Based on the above facts, the effective time of Huperzine A was significantly larger than neostigmine. The frequency of the various side effects, especially muscle bundle quivering, dizziness, perspiration, and blurred vision; Huporzine A was statistically lower than neostigmine.

Based on the above data on this group of 128 patients, it can be considered that Huperzine A is an effective anticholinesterase drug for treating myasthenia gravis. It did not have any significant negative effects on the major organs, for example, lungs, kidney, heart and the hematopoietic systems, and the clinical occurrence rate of side effects was low. Aside from nausea, it had lower side effects in all other areas than neostigmine. Moreover, the fact that its curative effect action time was noticeably longer than that of neostigmine is its major outstanding feature.

TABLE 10

				Comparison of the side effects between neostigmine and Huperzine A																									
				(1) %																									
				(7)		(8)		(9)		(10)		(11)		(12)		(13)		(14)		(15)		(16)		(17)		(18)		(19)	
(33)	(3)	(2)		(20)	%	(21)	%	(22)	%	(23)	%	(24)	%	(25)	%	(26)	%	(27)	%	(28)	%	(29)	%	(30)	%	(31)	%	(32)	%
(34)	39	356	(4)	37	10.4	72	20.3	13	3.7	25	7.0	46	12.9	21	5.9	10	2.8	9	3.5	3	0.8	13	3.0	11	3.1	10	2.8	11	3.2
	69	349	(5)	29	8.3	68	19.5	14	4.0	43	12.1	15	4.3	26	7.4	6	1.2	11	3.2			12	3.4			7	2.0	34	9.7
(35)	128	1226	(6)	49	4.0	100	8.1	38	3.1	59	4.8	95	7.8	80	6.5	15	1.2	25	2.0	5	0.4	13	1.0	13	1.0	11	0.5	22	1.8

Key: (1) % of side effect (2) number of operations (3) Number of cases (number of injections), (4) 39 cases used Huperzine A 355 times, (5) 69 cases used neostigmine 349 times, (6) 128 cases used Huperzine A 1,225 times, (7) Muscle bundle quivering; (8) Dizziness; (9) Tinnitus; (10) Perspiring; (11) Nausea; (12) Abdominal pains; (13) Vomiting; (14) Drowsiness; (15) Disturbed sleep; (16) Myosis; (17) Changes in the rhythm of the heart; (18) Increase in the number of bowel movements; (19) Blurred vision; (20)-(32) Number of times; (33) Group; (34) Control group; (35) Non-control group

Based on the fact that Huperzine A possesses definite pharmacodynamic activity and a relatively large therapeutic index, it was clinically tested. The results of the treatment of 128 cases with myasthenia gravis showed that the intramuscular injections of 0.4 mg. of Huperzine A were able to definitely improve the myasthenia gravis condition of the patients. Its sustained time of action was longer than that of neostigmine and it had lower side effects. The intramuscular injections of 25 or 50 µg of Huperzine A in 58 cases of cerebral arteriosclerosis accompanied by senile dementia was effective in improving memory functions.

A compound of formula I, II or III, or a salt thereof, or a composition containing a therapeutically effective amount of a compound of formula I, II or III, or a salt thereof can be administered by methods well known in the art. Thus, a compound of formula I, II or III, or a salt thereof can be administered either singly or with other pharmaceutical agents, for example, orally, parenterally or rectally. For oral administration they can be administered in the form of tablets, capsules, for example, in admixture with talc, starch, milk sugar or other inert ingredients, that is, pharmaceutically acceptable carriers, or in the form of aqueous solutions, suspensions, elixirs or aqueous alcoholic solutions, for example, in admixture with sugar or other sweetening agents, flavoring agents, colorants, thickeners and other conventional pharmaceutical excipients. For parenteral administration, they can be administered in solution or suspension, for example, an aqueous or peanut oil solution or suspension using excipients and carriers conventional for this mode of administration.

In the practice of the invention, the dose of a compound of formula I, II or III, or a salt thereof to be administered and the frequency of administration will be dependent on the potency and duration of activity of the particular compound of formula I, II or III, or salt to be administered and on the route of administration, as well as the severity of the condition, age of the mammal to be treated and the like. Doses of a compound of formula I or a salt thereof contemplated for use in practicing the invention for the treatment of myasthenia gravis are in the range of from about 0.01 to about 25 mg per day, preferably about 0.1 to about 10 mg either as a single dose or in divided doses, and for the treatment of senile dementia are in the range of from about 0.10 to about 100 mg. per day, preferably about 1.0 to about 50 mg. either as a single dose or in divided doses.

The Examples which follow further illustrate the invention. All temperatures are in degrees centigrade, unless otherwise stated.

EXAMPLE 1

Isolation of (5R, 9R, 11E)-5-amino-11-ethylidene-5,6,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one (Huperzine A)

About 100 kg dry weight of the crushed, powdered plant: Huperzia serrata (Thunb.) Trev., was placed in a container, and extracted with refluxing 95% ethanol several times. The combined ethanol extracts were evaporated to a residue which was suspended in dilute aqueous hydrochloric acid (1–2%) and extracted with ethyl ether to remove impurities. The aqueous layer was then neutralized with concentrated aqueous ammonia and the total alkaloids were extracted into chloroform. After partially concentrating the chloroform solution, the solution was repeatedly extracted with 1% sodium hydroxide. The sodium hydroxide layer was then neutralized with concentrated hydrochloric acid, and again brought back to pH greater than 10 with concentrated ammonia. This aqueous solution was extracted with chloroform and the residue from the chloroform extracts was chromatographed on silica gel column. Solvent system used was chloroform-methanol, 98:2; 97:3; and 96:4 ratio in succession. Fractions from the chromatography were analyzed by TLC and those with a single spot were combined. After solvent removal, the residue was crystallized from acetone to give crude (5R, 9R, 11E)-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one (Huperzine A), about 10 g; yields ran 0.005% to 0.011% of starting dry powdered plant.

The crude Huperzine A was analyzed to be about 95% pure or better and contained about 2% (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one (Huperzine B). This material with a mp of 221°–229° C., was used in clinical trials.

To further purify Huperzine A, the crude material was rechromatographed using the chloroform: methanol solvent mixture or recrystallized from methanol/acetone mixture. The pure material has mp 230° C.

m. wt. $C_{15}H_{18}N_2O$; 242.1426 (By mass spectroscopy).

$[\alpha]_D^{25}$ − 150.4° (conc. 0.498 in methanol).

UV max. (ethanol) 231 nm (log ε 4.01); 313 nm (log ε 3.89).

IR: 1650, 1550, 3480, 3340, 3269 cm−1.

EXAMPLE 2

Isolation of (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one (Huperzine B)

The crude material isolated from later fractions of the chromatograph column was found to be a minor component. Further purification involved rechromatographing on silica gel using a solvent system of chloroform-acetone-methanol in 50:47:3 ratio. The material collected from the column was recrystallized from acetone to give pure (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one (Huperzine B). m.p. 270°-271° C.

m. wt. $C_{16}H_{20}N_2O$: 256.1558 (by mass spectroscopy).
$[\alpha]_D^{22}$ − 54.2° (conc. 0.203% in methanol).
Yield 0.000833% based on dry plant (8.33×10^{-6}).

EXAMPLE 3

Preparation of (5R, 9R, 11E)-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5-(methylamino)-5,9-methanocycloocta[b]pyridin-2(1H)-one

The mono-methyl derivative of Huperzine A was prepared from Huperzine A (150 mg.) by the treatment with methyl iodide (1 ml.) in methanol (0.5 ml.) and acetone (2 ml.) overnight. After concentrating, product was recrystallized from acetone (yield 120 mg.)

mp 235°-236° C.
MS 256 (M⁺).

EXAMPLE 4

Preparation of (5R, 9R, 11E)-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5-(dimethylamino)-5,9-methanocycloocta[b]pyridin-2(1H)-one

The di-methyl derivative of Huperzine A was obtained by the treatment of Huperzine A (150 mg.) with formic acid (88%, 1 ml.) and formaldehyde (35%, 1 ml.) at 100° C. for 4 hours. After concentrating under reduced pressure and basifying with conc. ammonium hydroxide, the desired product was extracted with chloroform. Recrystallization from a chloroform-methanol mixture gave pure title compound (yield 150 mg.).

mp 243°-245° C.
MS 270 (M⁺).

EXAMPLE 5

Preparation of (5R, 9R, 11E) 11-ethylidene-5,6,9,10-tetrahydro-1,7-dimethyl-5-(dimethylamino)-5,9-methanocycloocta[b]pyridin-2(1H)-one

The title trimethyl derivative of Huperzine A was obtained by methylation of Huperzine A (150 mg.) with dimethyl sulfate (3 ml.) in acetone (10 ml.) and 20% aqueous sodium hydroxide (4 ml.) at reflux. After three (3) hours, the mixture was extracted with chloroform. TLC analysis of this extract showed two spots. Purification by silica gel column chromatography (chloroform as solvent, impurity being eluted first) gave the trimethyl derivative as an oil (yield 110 mg.). The title compound is an oil.

MS 284 (M⁺).

EXAMPLE 6

Preparation of (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-1,12-dimethyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one

Methylation of Huperzine B (150 mg) according to the method as utilized in Example 4 gave (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-1,12-dimethyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one, recrystallized from methanol (yield 150 mg.).

m.p. 272°-273° C.
MS 270 (M⁺).

EXAMPLE 7

Preparation of (4aR, 5R, 10bR, 12S)-1,2,3,4,4a,5,6,10b-octahydro-1,12-dimethyl-10b,5-propano-1,7-phenanthrolin-8(7H)-one

Monomethyl Huperzine B (140 mg.) was hydrogenated in the presence of platinum oxide (100 mg.) and acetic acid (5 ml.). After basification with ammonium hydroxide and extraction into chloroform, the title product was recrystallized from chloroform-methanol (yield 130 mg.).

m.p. 281°-3° C.
MS 272 (M⁺).

EXAMPLE 8

Preparation of (5R, 9R)-5-amino-11-ethyl-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one

Hydrogenation of Huperzine A (150 mg.) in the presence of platinum oxide (60 mg.) in ethanol (20 ml.) gave the title dihydrohuperzine A, where the former exo-double bond is saturated. This material was purified by silica gel column chromatography (chloroform-methanol, 15:1 as solvent) followed by recrystallization from methanol-acetone (yield 100 mg).

m.p. 269°-270° C.
MS 244 (M⁺).

EXAMPLE 9

Preparation of (5R, 9R)-5-amino-11-ethyl-5,6,7,8,9,10-hexahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one

Huperzine A (200 mg.) was hydrogenated in the presence of platinum oxide (100 mg.) and acetic acid (10 ml.). After basification and extraction into chloroform, the title tetrahydrohuperzine A was recrystallized from a methanol-acetone mixture (yield 180 mg.).

m.p. 264°-5° C.
MS 246 (M⁺).

EXAMPLE 10

Preparation of (5R, 9R, 11E)-5-(acetylamino)-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one

The titled N-acetyl Huperzine A derivative was prepared by treating Huperzine A (100 mg.) with acetic anhydride (1 ml.) and pyridine (0.5 ml.) at room temperature for one week. This mixture was poured into ice-water and extracted with chloroform. The chloroform extract was concentrated and purified by silica gel column chromatography (chloroform-methanol, 15:1 as solvent) and recrystallization from acetone (yield 100 mg.).

m.p. 276°-7° C.
MS 284 (M+).

EXAMPLE 11

An injection of the following composition is prepared in the usual manner:

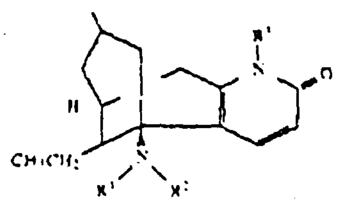
(5R,9R,11E)-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one hydrochloride	50 mg.
Water for injection q.s. ad	2.00 ml.

We claim:

1. Essentially pure (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one.

2. A pharmaceutically acceptable acid addition salt of (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one.

3. A compound of the formula



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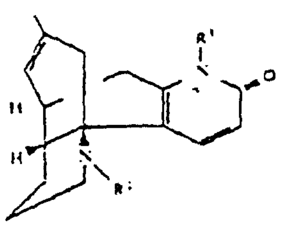
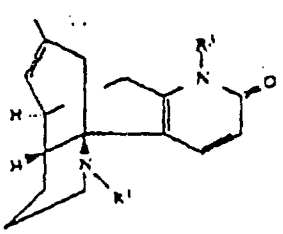


III

wherein R^1, R^2 and R^3 independently are hydrogen or lower alkyl, the dotted (. . .) line is an optional double bond, and provided that in formula III one of R^1, R^2 and R^3 is other than hydrogen, or a pharmaceutically acceptable acid addition salt thereof.

4. A compound, in accordance with claim 3, of the formula

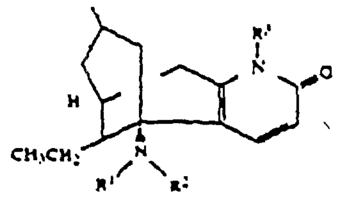


III

wherein R^1 and R^2 independently are hydrogen or lower alkyl, and provided that one of R^1 and R^2 is other than hydrogen, or a pharmaceutically acceptable acid addition salt thereof.

5. A compound, in accordance with claim 4, (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-1,12-dimethyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one.

6. A compound, in accordance with claim 3, of the formula



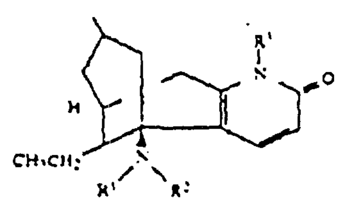
II

wherein R^1, R^2, and R^3 independently are hydrogen or lower alkyl, and the dotted (. . .) line is an optional double bond, or a pharmaceutically acceptable acid addition salt thereof.

7. A compound, in accordance with claim 6, (5R, 9R)-5-amino-11-ethyl-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one.

8. A compound, in accordance with claim 6, (5R, 9R)-5-amino-11-ethyl-5,6,7,8,9,10-hexahydro-7-methyl-5,9-methanocycloocta[b]pyridin-2(1H)-one.

9. A pharmaceutical composition comprising an effective amount of an essentially pure compound of a formula



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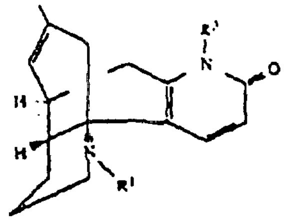

III

wherein R^1, R^2 and R^3 independently are hydrogen or lower alkyl, and the dotted (. . .) line is an optional double bond, or a pharmaceutically acceptable acid addition salt thereof and an inert pharmaceutical carrier.

10. A pharmaceutical composition, in accordance with claim 9, wherein the compound is (4aR, 5R, 10bR)-1,2,3,4,4a,5,6,10b-octahydro-12-methyl-5,10b-propeno-1,7-phenanthrolin-8(7H)-one.

* * * * *

Section 4

Huperzine A

Pharmacokinetics of tablet huperzine A in six volunteers[1]

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AIM: To study pharmacokinetics of tablet huperzine A (Hup-A) in Chinese volunteers to help establishing its drug administration schedule. **METHODS**: For 6 volunteers after a single oral dose of 0.99 mg, drug concentrations in plasma were assayed by reverse phase high pressure liquid chromatography (HPLC) at 0.5, 0.75, 1.0, 1.25, 1.5, 2, 4, 6, 8, and 10 h. The pharmacokinetic parameters were calculated with a 3P87 program by computer. **RESULTS**: The time course of plasma concentrations conformed to a one-compartment open model with a first order absorption. The pharmacokinetic parameters were as follows: $T_{\frac{1}{2}k_a} = 12.6$ min, $T_{\frac{1}{2}k_e} = 288.5$ min, $T_{max} = 79.6$ min, $C_{max} = 8.4$ μg L^{-1}, AUC = 4.1 mg L^{-1} min. **CONCLUSION**: Hup-A was absorbed rapidly, distributed widely in the body, and eliminated at a moderate rate.

KEY WORDS huperzine A; cholinesterase inhibitors; high pressure liquid chromatography; pharmacokinetics; phase I clinical trials

Huperzine A (Hup-A), a new alkaloid first isolated from Chinese herb *Huperzia serrata* (Thunb) Trev[1], exhibited a selective inhibition on acetylcholinesterase (AChE)[2]. It potentiated the skeletal muscle contraction and increased muscle tones[3], and enhanced rodent learning and memory[4]. Clinically, Hup-A improved muscle weakness of myasthenia gravis[5] and memory in patients with impaired memory or Alzheimer's disease[6]. The plasma level of Hup-A following iv or ig [^{3}H]Hup-A 13.9 MBq kg^{-1} in rats declined in two phases, the distribution phase and the elimination phase, with half-lives of 6.6, 149 min (iv) and 10, 203 min (ig) respectively[7]. This paper was to study the pharmacokinetics of Hup-A in healthy volunteers to help establishing its drug administration schedule in clinic.

MATERIALS AND METHODS

Drug According to Chinese National Standard tablet Hup-A (batch № 940112) was prepared by the Institute of Materia Medica, Zhejiang Academy of Medical Sciences. The purity of Hup-A was 99.5 %. Each tablet contains Hup-A 0.09 mg. (±)-Dinor Hup-A as internal standard was synthesized and presented kindly by Dr HE Xu-Chang, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and 3 mg L^{-1} was used for experiment.

Subjects Six Chinese volunteers (M 3, F 3), aged 27±6 a and weighing 58±7 kg were all healthy, not in pregnant or menstruation. Each volunteer was told about the aim and process of the study. Agreements were obtained from them before study. Each subject was given a single oral dose of 0.99 mg Hup-A tablet at 8 am after an overnight fasting. Breakfast was served at 10 am. Blood (5 mL) was collected from an indwelling catheter in antecubital vein before and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, 4, 6, 8, and 10 h after *po*. Plasma (2 mL) was taken for HPLC. Pharmacokinetic parameters were obtained by first calculating the parameters from each person and then taking average of the 6 parameters, using a 3P87 program provided by Chinese Mathematic-Pharmacological Society on the computer.

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HPLC Shimadzu LC-6A liquid chromatography was connected to SPD-6A uv spectrophotometric detector (Shimadzu) and Rheodyn 7125 sampler, recorded on C-R3A integrator (Shimadzu). The column was a Spherisorb C18 (150 mm × 5 mm inner diameter; 5 μm particle size). The mobile phase was methanol: water (45:55, vol/vol), 1.0 mL min^{-1} at 30 ℃ column oven. The column effluent was monitored at 313 nm.

Plasma sample Add (±)-dinor Hup-A 100 μL to plasma 2 mL, add Na_2CO_3-$NaHCO_3$ buffer 1 mL (using NaOH 1 mol L^{-1} to adjust pH to 11.9). Then add chloroform 7.5 mL, shake 2 min, and centrifuge at 1000 × *g* for 10 min. The organic phase was blown to dryness by N_2 at 40 ℃. Dissolve the residue with HPLC mobile phase 50 μL, and 20 μL was applied to HPLC. Hup-A peak and (±)-dinor Hup A peak were separated clearly. The retention times (Rt) of (±)-dinor Hup-A and Hup-A were 3.5 and 8.3 min, respectively (Fig 1).

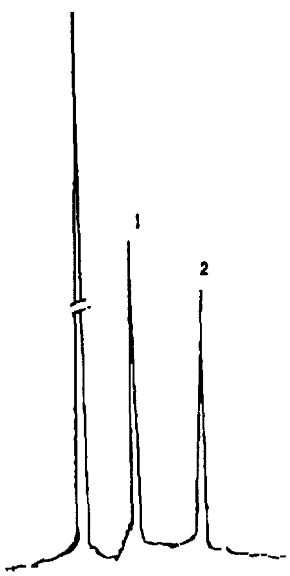

Fig 1. Chromatogram of blank plasma spiked with internal standard (peak 1, retention time 3.5 min) and Hup-A (peak 2, retention time 8.3 min).

Standard curve To the plasma containing (±) dinor Hup-A add Hup-A 2.20, 4.43, 7.08, 8.85, and 17.70 μg L^{-1}, according to the ratio of Hup-A peak area to (±) dinor Hup-A peak area in HPLC, a linear equation $\hat{Y} = 0.0188X - 0.0069$ was obtained ($r = 0.9988$). The minimal detect limit of plasma Hup-A was 1.60 μg L^{-1}. The recovery of Hup-A was 95.7 ± 5.5 % ($n = 9$) and coefficient of variation was 6.4 %. According to measurements of 3 standard plasma Hup-A concentrations, intraday and interday variances were 5.5 %–7.4 % ($n = 9$) and 6.0 %–9.9 % ($n = 9$), respectively.

RESULTS

The plasma concentrations of Hup-A after oral administration of 0.99 mg within 10 h were fitted well to a one-compartment open model with a first order absorption (Fig 2).

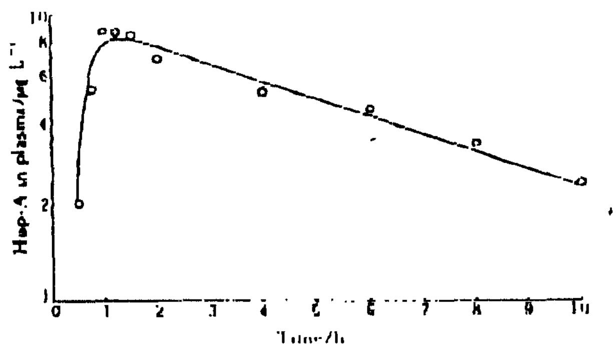

Fig 2. Mean plasma concentration-time curve after *po* tablet Hup-A 0.99 mg in 6 adults.

Hup-A was absorbed quickly after *po* with $T_{\frac{1}{2}k_a} = 12.6$ min and time peak for plasma averaged 79.6 min. It indicated that Hup-A was released and absorbed quite well *in vivo*. Plasma mean peak concentration after *po* was 8.4 μg L^{-1}, V_d/F was 0.108 L kg^{-1}, indicating that Hup-A was widely distributed *in vivo*. Mean elimination half life $T_{\frac{1}{2}k_e}$ was 288.6 min, suggesting that Hup A have a mild elimination rate (Tab 1).

DISCUSSION

Hup-A showed some advantages, compared with the first generation of ChE inhibitors such as physostigmine (Phy) and tetrahydroaminoacridine (THA). LD_{50} value in mice for Hup-A ip was 1.8 mg kg^{-1} and

Tab 1. Pharmacokinetic parameters of Hup-A after po tablet 0.99 mg in 6 healthy volunteers. $\bar{x}\pm s$.

Parameter		$\bar{x}\pm s$
K_a	min^{-1}	0.061±0.017
K_e	min^{-1}	0.0025±0.0006
$T_{\frac{1}{2}K_a}$	min	13±5
$T_{\frac{1}{2}K_e}$	min	288±63
T_{max}	min	80±9
C_{max}	$\mu g\ L^{-1}$	8.4±0.9
T_{lag}	min	25.4±1.8
V_d/F	$L\ kg^{-1}$	0.108±0.008
AUC	$mg\ L^{-1}\ min$	4.1±1.2

that for Phy was 0.6 mg kg^{-1}[4]. Hup-A at optimal doses has a long term inhibition of AChE in rat brain (up to 360 min) and only 60 min for Phy[9]. The results of this paper showed that in human being $T_{\frac{1}{2}\beta}$ of Hup-A was 288.5 min. However, for Phy the $T_{\frac{1}{2}\beta}$ was 20 min[8]. Hup-A was absorbed rapidly, distributed widely in the body and eliminated at a middle rate[7]. Therefore it is better to take tablet Hup-A orally 2—3 times a day.

As a new ChE inhibitor, Hup-A shows some interesting cholinomimetic properties and its effects satisfy more closely established criteria for therapeutic use than effects of previously tested compounds. Hup-A is a new promising ChE inhibitor.

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石杉碱甲片在六名志愿者体内的药物动力学

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(浙江省医学科学院药物研究所，杭州310013，中国)

目的：了解石杉碱甲片在人体内的药物动力学过程，为设计临床用药方案提供依据. 方法：用反相高效液相色谱法测定六名健康志愿者口服片剂0.99 mg后的血药浓度，按3P87程序计算动力学参数. 结果：石杉碱甲片在体内的药时过程符合一级吸收的一室开放模型. 主要动力学参数：$T_{\frac{1}{2}K_a}$ 12.6 min，$T_{\frac{1}{2}K_e}$ 288.5 min，T_{max} 79.6 min，C_{max} 8.4 $\mu g\ L^{-1}$，AUC 4.1 mg L^{-1} min. 结论：石杉碱甲吸收迅速，属于中等速率消除类药物.

关键词 石杉碱甲；胆碱酯酶抑制剂；高压液相色谱法；药物动力学；I期临床试验

Structure of acetylcholinesterase complexed with the nootropic alkaloid, (–)-huperzine A

Mia L. Raves[1], Michal Harel[1], Yuan-Ping Pang[2], Israel Silman[3], Alan P. Kozikowski[4] and Joel L. Sussman[1,5]

(–)-Huperzine A (HupA) is found in an extract from a club moss that has been used for centuries in Chinese folk medicine. Its action has been attributed to its ability to strongly inhibit acetylcholinesterase (AChE). The crystal structure of the complex of AChE with optically pure HupA at 2.5 Å resolution shows an unexpected orientation for the inhibitor with surprisingly few strong direct interactions with protein residues to explain its high affinity. This structure is compared to the native structure of AChE devoid of any inhibitor as determined to the same resolution. An analysis of the affinities of structural analogues of HupA, correlated with their interactions with the protein, shows the importance of individual hydrophobic interactions between HupA and aromatic residues in the active-site gorge of AChE

(–)-Huperzine A (HupA), an alkaloid isolated from the club moss, *Huperzia serrata*, which has found use in Chinese herbal medicine[1], is a potent reversible inhibitor of acetylcholinesterase (AChE) that lacks potentially complicating muscarinic effects[2,3]. Its unusual pharmacological properties raise the possibility that HupA may be used in symptomatic treatment of disorders believed to involve cholinergic insufficiency[4]. In particular, there is substantial evidence for a role for acetylcholine (ACh) in learning and memory[5], and the cholinergic hypothesis postulates that a cholinergic deficit in Alzheimer's Disease (AD) may be alleviated by cholinesterase inhibitors[6]. Although one AChE inhibitor, tacrine, has been licensed for use in patients with AD[7], and others are at various stages of clinical evaluation[8], the existence of a natural AChE inhibitor, taken together with its unique pharmacological features and relative lack of toxicity[9], render HupA a particularly promising candidate for AD treatment.

Studies on experimental animals reveal significant cognitive enhancement[10], and clinical trials in China have both established the safeness of HupA and provided preliminary evidence for significant effects on patients exhibiting dementia and memory disorders[11]. It was recently demonstrated that HupA decreases neuronal cell death caused by glutamate, particularly in primary cultures derived from hippocampus and cerebellum of the embryonic rat[12]. Its dual pharmacological action suggests that HupA may be a unique and important drug for the treatment of AD patients, since it may serve both to alleviate reduced ACh levels in the brain and to decrease neuronal cell death.

The structure of racemic Huperzine A[13] shows some similarity to other known AChE inhibitors. The molecule is fairly rigid and contains an aromatic system as well as a primary amino group that is probably protonated at physiological pH[14]. It is an optically active molecule, with the naturally occurring (–)-HupA being the more potent of the two enantiomers for both mammalian and *Torpedo californica* AChE (TcAChE)[15,16]. It binds reversibly to fetal bovine serum AChE with a dissociation constant of 6 nM. The affinity for TcAChE is 40-fold lower (K_i=250 nM), and binding to human butyrylcholinesterase is four orders of magnitude weaker (K_i=76 μM)[16].

Visual inspection of HupA reveals no immediate similarity to ACh (Fig. 1). Indeed, various suggestions have been made with respect to its orientation within the active site of AChE, and with respect to the amino acid residues with which its putative pharmacophoric groups might interact[14,16,17]. Solution of the 3D

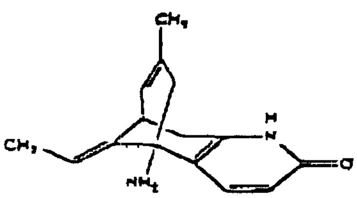

Fig. 1 Structure diagram of naturally active Huperzine A. The molecule is chiral, with the (–)-isomer being more potent than either the (+)-isomer or the racemic mixture.

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[2]The Mayo Clinic, Jacksonville, Florida 32224, USA
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[5]Department of Biology, Brookhaven National Laboratory, Upton, New York 11973-5000, USA

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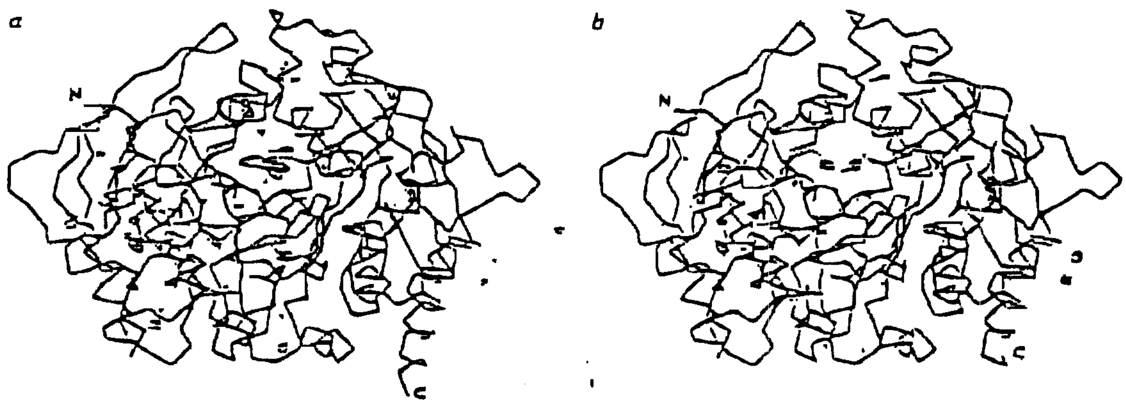


Fig. 2 Cα trace of AChE a, in the HupA complex and b, in the native protein, showing the initial F_o–F_c maps with 3.0 σ cut-off for all data from 20.0–2.5 Å resolution. The electron density for the HupA molecule is clearly the most prominent feature of the difference map of the complex. Both maps were calculated after a sequence of (i) molecular replacement using the 1ACE structure (resolution 2.5 Å) as a model, (ii) rigid-body and positional refinement, (iii) simulated annealing at 3000 K and (iv) B-factor refinement.

structure of a complex of HupA with AChE would permit unequivocal resolution of this issue. Furthermore, it would provide a rational basis for structure-related drug design aimed at developing synthetic analogues of HupA with improved therapeutic properties.

In the following, we report the solution of the structure of a complex of HupA with TcAChE to 2.5 Å resolution, which permitted us to determine the correct orientation and interactions of HupA within the active-site gorge. In addition, the structure of the native enzyme was determined at the same resolution so as to permit accurate pinpointing of the changes in the protein structure brought about by the binding of HupA. The structure of the complex allowed the rationalization of the reported differences in affinity of the ligand for cholinesterases from different species, as well as the different affinities of structural analogues of HupA for AChE.

Structure determination

The highest peak in the initial F_o–F_c maps of the AChE–HupA complex, and two other peaks, at 7.1, 5.6 and 5.6 σ, respectively, were located near the active site, at the bottom of the aromatic gorge. A molecule of HupA was placed in the density around these positions, which roughly resembles its outline. 208 waters were located in F_o–F_c maps, one of which is positioned on the crystallographic two-fold axis, as well as three C-terminal residues that were previously undetermined[11], including Cys 537, which forms a disulphide bond between the two monomers across the crystallographic two-fold axis. The final R-factor for the refined structure of the AChE–HupA complex is 20.5% for all data between 8.0–2.5 Å, and R_{free} = 24.5%. The r.m.s. deviations from ideality are 0.015 Å in bond lengths and 1.9° in bond angles. Electron density difference maps for the native structure show clearly that the active site of the enzyme is devoid of inhibitor. The native AChE yielded a residual R-factor of 19.9% on refinement, with R_{free} = 25.3% and 204 waters, including one on the crystallographic two-fold axis. The r.m.s. deviations from ideality for the native structure are 0.014 Å in bond lengths and 1.98° in bond angles.

The initial unbiased F_o–F_c map for the AChE–HupA complex is shown in Fig. 2a, alongside a similar map for the native structure (Fig. 2b). From comparison of the two maps it is evident that the only prominent electron density in the difference map of the complex is located near the bottom of the active-site gorge[18], with an outline resembling that of HupA. A close-up of this density, displayed at 4.0 σ cutoff, is shown in Fig. 3, with the refined structure of HupA superimposed. Excellent fitting of the molecule to the electron density can be seen.

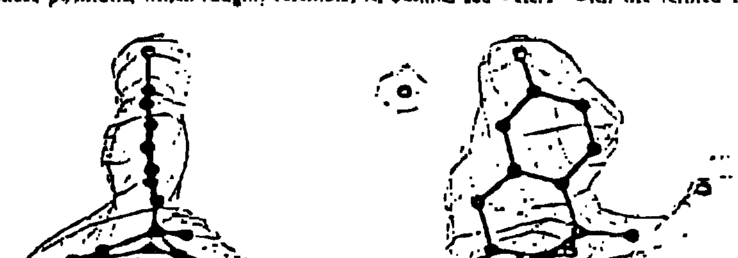


Fig. 3 Two views of the refined structure of HupA in the active site of AChE displayed in the initial F_o–F_c map with 4.0 σ cut-off, showing the excellent fit of the molecule to the electron density.

Protein–ligand interactions

The principal protein–ligand interactions revealed by the refined structure are displayed schematically in Fig. 4. These include: (i) a strong hydrogen bond (2.6 Å) of the carbonyl group of the ligand to the hydroxyl oxygen of Tyr 130; (ii) hydrogen bonds to water molecules within the active-site gorge which are, themselves, hydrogen-bonded to other waters or to side-chain and backbone atoms of the protein, notably to carboxylic oxygens of Glu 199 and to the hydroxyl oxygen of Tyr 121; (iii) interaction of the primary

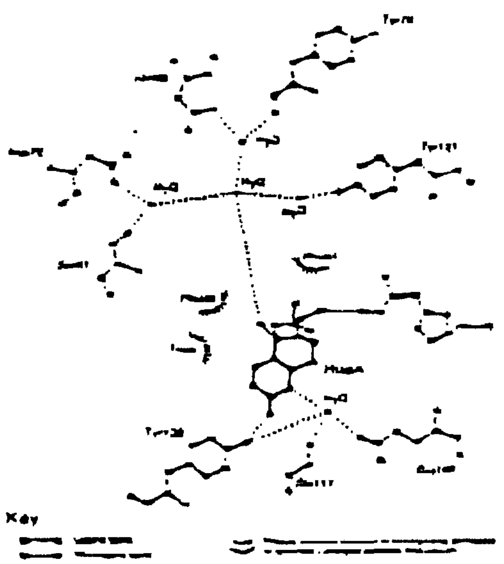


Fig. 4 Schematic figure (using LigPlot[45]) showing the main interactions between the protein and the ligand.

amino group of the ligand, which can be assumed to be charged at the pH of the mother liquor (pH 5.8)[14], with the aromatic rings of Trp 84 and Phe 330, with distances between the nitrogen and the centroids of the rings of 4.8 and 7.7 Å, respectively—this interaction is analogous to that observed for the primary amino group of tacrine[17]; (iv) an unusually short (3.0 Å) C–H···O hydrogen bond between the ethylidene methyl group and the main-chain oxygen of His 440 (a member of the catalytic triad); and (v) several hydrophobic contacts, notably with the side chains and main-chain atoms of Trp 84 and His 440, and with residues Gly 118 through Ser 122.

Fig. 5a shows the initial electron-density difference map of HupA within the active-site gorge and the surrounding protein residues. Fig. 5b shows the putative waters present in the gorge in the native structure, before the ligand was soaked in, superimposed on the HupA electron density. It can be seen that seven of these waters roughly occupy the place of the ligand. The surrounding residues of the protein retain essentially their original position and orientation.

Native structure

The native structure of TcAChE determined to a higher resolution (2.3 Å) than the original structure (2.8 Å)[18] is, in fact, of interest not only due to the higher accuracy. The original structure (PDB entry code 1ACE) still contains within its active-site gorge a significant amount of the bisquaternary inhibitor, decamethonium, used for elution from the affinity chromatography resin[20]. Although the presence of various inhibitors within the crystal structure of AChE does not cause a large change in the overall conformation of the protein[19-21], some of the differences observed, especially in the conformations of the side chains of aromatic residues, may be significant. In the present paper, the AChE preparation from which the crystals were obtained was eluted from the affinity resin with the small monoquaternary ligand tetramethylammonium; the electron density ascribed to decamethonium is no longer apparent.

However, the highest peak in the initial electron-density difference map is located at 4.2 Å from the indole ring of Trp 84, close to the position of the proximal quaternary group of decamethonium (PDB access code 1ACL). It is possible that this peak (X0 in Fig. 5b) is a (partially occupied) tetramethylammonium or other cationic species, rather than a water molecule. It is of interest that two other prominent peaks in the difference map (X2 and X3, Fig 5b), correspond to two of the putative cations (2* and 3* respectively) proposed earlier by Axelsen et al.[16] on the basis of molecular dynamics studies. The third putative cation, 1*, is also observed in the structure, but is not in the immediate vicinity of the HupA density and therefore not included in Fig. 5b. Wlodek et al.[22], on the basis of electrostatic calculations, have suggested that a small cation must be present in the active site of AChE for the enzyme to function. Our structural data are in agreement with these independent theoretical studies which point to a structural and/or functional role for small cations in the active site of AChE.

Comparison of the native coordinates with those for the 1ACE structure shows that the structures are very similar; the r.m.s. deviation in the position of the Cα atoms in the two structures is 0.31 Å. There is only one difference in the configuration of the protein backbone, in the orientation of the peptide bond between residues Pro 163 and Gly 164, which is probably just due to the higher resolution of the maps. A few side chains display a change in conformation, notably Phe 330, in the active site (from $\chi 1 = -116°$, $\chi 2 = -65°$ to $\chi 1 = -160°$, $\chi 2 = -85°$), and Trp 279, at the peripheral anionic site (from $\chi 2 = 114°$ to $\chi 2 = 91°$). These differences are significant, because decamethonium interacts with both residues, influencing their conformations[17]. A few other large side chains, that are not close to the active site, have a slightly different conformation. Based on all

Table 1 Data collection and processing statistics

	HupA	native
Oscillation angle	1.0°	1.0°
No. of frames	90	130
Total no. of observations	334,827	138,332
Average redundancy	5.3	1.3
No. of independent refl.	44,503	45,243
Highest resolution processed*	2.3 Å	2.25 Å
Highest resolution in refinement	2.5 Å	2.3 Å
Completeness	99.3%	96.6%
highest resolution shell	99.6% (2.35–2.3 Å)	96.7% (2.33–2.25 Å)
highest resolution used in refinement	100.0% (2.55–2.48 Å)	99.1% (2.47–2.53 Å)
R_{sym}	9.6%	9.5%
highest resolution shell	57.3% (2.35–2.3 Å)	62.1% (2.33–2.25 Å)
highest resolution used in refinement	33.6% (2.55–2.48 Å)	32.3% (2.67–2.53 Å)
Average I / average σ	23.7	7.4
highest resolution shell	3.0 (2.35–2.3 Å)	0.9 (2.35–2.25 Å)
highest resolution used in refinement	5.3 (2.55–2.48 Å)	2.1 (2.67–2.53 Å)

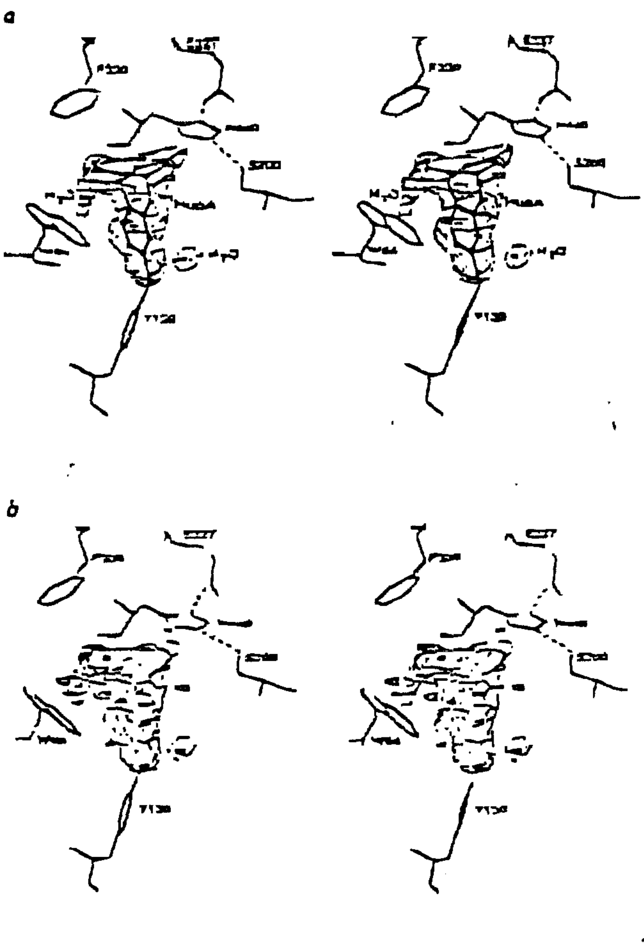


Fig. 5 Stereo views of the initial F_o-F_c map at 4.0 σ around HupA with: a, the refined structure of the HupA–AChE complex and two waters in the active site of AChE superimposed; b, the same residues in the 2.5 Å native structure and several putative waters in the active site superimposed. K0 roughly occupies the position of the proximal quaternary group of decamethonium (PDB access code 1ACL). K2 and K3 correspond to two of the sites earlier assigned as possible cations[19]. The hydrogen bond between HupA and Tyr 130, and those in the catalytic triad (Ser 200, His 440 and Glu 327) are shown with dashed lines.

inhibitor complexes determined so far, it seems that Phe 330 is the most flexible residue in the protein, as indicated also by the smeared appearance of the electron density for its side chain in the high-resolution native structure.

Docking studies

In our original report on the 3D structure of AChE, we suggested a plausible orientation, obtained by manual docking, of ACh in an all-trans conformation within the active site[1]. In this model, the quaternary ammonium group is positioned directly above the six-membered ring of Trp 84, at 4.1 Å from the nitrogen atom to the centroid of the ring, and at 3.9 Å from Phe 330. In the 2.5 Å native structure, we oriented the modelled ACh molecule so that the acetate moiety retains its position near Ser 200, and the quaternary nitrogen is positioned at 4.5 Å from the centroid of the entire nine-membered indole ring (as the partial charge of the aromatic system is distributed over all nine atoms[20]), so that the distance to the phenyl ring of Phe 330 is now 3.2 Å. Thus, the quaternary ammonium group makes two cation–aromatic interactions. The validity of this model is supported by our X-ray studies of complexes of the enzyme with the reversible inhibitor, edrophonium[19], and with the transition-state analogue, *m*-(*N,N,N*-trimethylammonio)trifluoroacetophenone[21].

Consideration of its pharmacophoric groups suggests a plausible orientation for HupA parallel to the ACh molecule[4] (Fig. 6a). But, in fact, its observed orientation within the active-site gorge appears to be almost orthogonal (Fig. 6b). This may explain why several orientations predicted by docking studies were erroneous[14,16]. Saxena *et al.*[16] proposed that the carbonyl group of HupA points towards the putative oxyanion hole and that the primary amino group may interact with the carboxylate of Glu 199. Ashani *et al.*[14] assigned the primary amino group, together with the endocyclic or exocyclic double bonds, as interacting with Trp 86 and Tyr 337 in human recombinant AChE (corresponding to Trp 84 and Phe 330 in *Tc*AChE), and suggested that the pyridone ring heteroatoms form hydrogen bonds to amino acids distal to Tyr 337.

In a docking study using the automated docking program, SYSDOC, three possible orientations of HupA within the active-site gorge were suggested[17]. Binding of HupA to the peripheral site, near Trp 279, was also predicted, but no evidence of that is found in the crystal structure, possibly because the tight packing of the AChE molecules within the crystal produces steric hindrance which precludes binding of ligands to the 'peripheral' site upon soaking[29]. One of the three candidate orientations differs only slightly from that of the crystal structure, inasmuch as it predicts that the pyridone oxygen should be bonded to the main-chain nitrogen of Ser 124 rather than to the hydroxyl group of Tyr 130, with the adjacent ring nitrogen hydrogen-bonding to this hydroxyl instead (Fig. 7). However, this orientation does imply a short distance between the pyridone oxygen and the hydroxyl group of Tyr 130 in the SYSDOC-generated complex.

Binding of HupA analogues

It seems surprising that an inhibitor with such a strong affinity for AChE as HupA binds by means of so few direct contacts. First, even though HupA has three potential hydrogen-bond donor and acceptor sites (Fig. 1), only one strong hydrogen bond is seen, between the pyridone oxygen of the ligand and a protein residue (Fig. 4). Analogous compounds with a methoxy group instead of the carbonyl oxygen show no inhibition at all (Table 2). Second, the ring nitrogen is hydrogen-bonded to the protein through a water molecule, and hydrogen bonds between the $-NH_3^+$ group and the protein are mediated by at least two

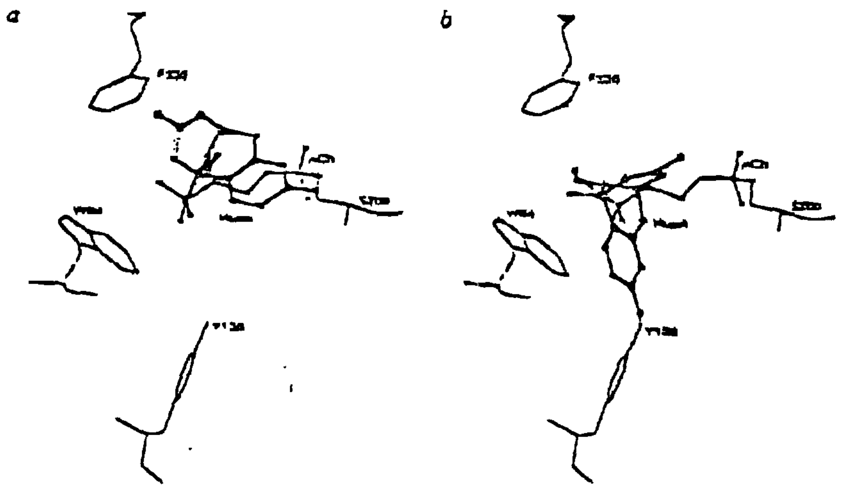


Fig. 6 Orientation of HupA in the active site a, based on similarity to ACh and b, in the X-ray structure. HupA is shown with thick balls-and-sticks. ACh is shown attached to Ser 200 in the tetrahedral intermediate state, with thin balls-and-sticks.

waters. Third, the aromatic rings of Trp 84 and Phe 330 are near the primary ammonium group, but do not have the preferred tangential orientation that was found in a study of small-molecule structures[25]. Modelling Phe 330 in the crystal structure as a tyrosine, which is the corresponding residue in mammalian AChE, permits the formation of a 3.3 Å hydrogen bond between the hydroxyl oxygen and the primary amino group of HupA. This extra hydrogen bond, together with the cation–π interactions, may explain why HupA binds to mammalian AChE five-to tenfold more strongly than to TcAChE, and only weakly to BuChE, which has no aromatic side chain at this position. Fourth, the short C–H·O hydrogen bond is somewhat unusual, but this type of bonding has previously been reported both in small molecules[26,27] and in proteins[28]. Moreover, the fact that analogue A3 (Table 2)—which differs from HupA only in lacking the methyl group that makes this hydrogen bond—has an affinity that is lower by two orders of magnitude, argues that the interaction is a favourable one, and serves to stabilize HupA in the active-site gorge. Fifth, the crystal structure of the complex shows a large number of hydrophobic interactions: there are 11 contacts between a carbon atom of HupA and oxygen or nitrogen atoms of protein residues and 20 carbon–carbon contacts within 4.0 Å. The exact location of the double bond in the ethylidene tail doesn't seem critical, since the activity of HupC is comparable to that of HupA (Table 2).

There does not appear to be much room for adding additional groups without causing clashes: there is some room near the bridge methyl group, which points into a highly aromatic environment, but not enough for an entire phenyl ring (analogue A7). However, based on modelling studies[29], it was predicted that the addition of a methyl group near the amide group of HupA (analogues A11 and A12, Table 2) should improve binding. Indeed, when this methyl group is in an axial position, the compound displays an eightfold increase in affinity, probably due to extra hydrophobic contacts with Trp 84.

The side chains of residues in the active-site gorge occupy almost identical positions in the native structure and in the complex, and the r.m.s. deviation of Cα atoms between the two structures is 0.30 Å. One major change is observed, however, in the orientation of the peptide bond between Gly 117 and Gly 118 in the so-called oxyanion hole, where the main-chain carbonyl oxygen atom of Gly 117 distinctly points in the opposite direction from that observed in the native structure and in other inhibitor complexes determined so far[17,21,30]. This change in the conformation of the main chain can only have been brought about by the binding of HupA, since the superposition of AChE in the native structure and in the complex shows that the carbonyl groups of Gly 117 and of HupA would be almost parallel if the movement were not to occur, with a close distance of 3.0 Å between the oxygen atoms. We suggest that this peptide flip[31] is

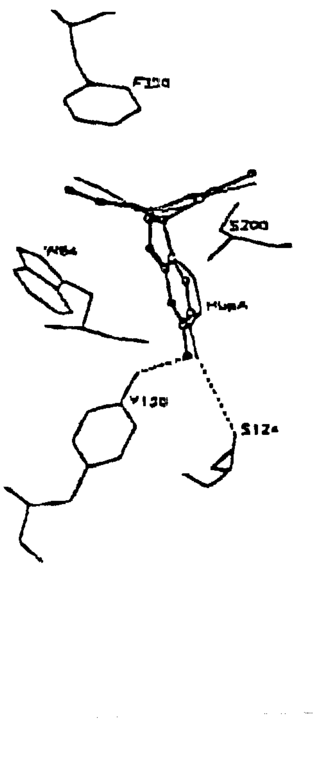
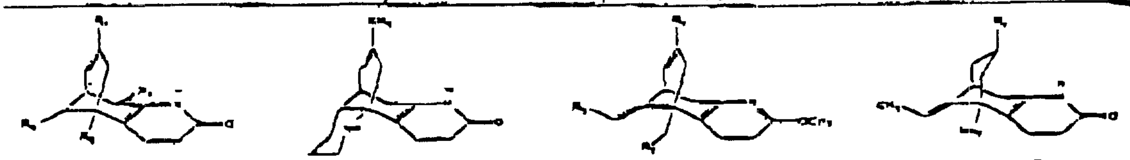


Fig. 7 Orientation of HupA at the active site of AChE obtained in the SYSDOC modelling study performed prior to the determination of the crystal structure of the AChE–HupA complex. The predicted structure (shown in magenta) is almost identical to the X-ray structure (thick balls-and-sticks). For clarity, the molecule in this figure has been rotated by 45° around the vertical (y) axis relative to the orientation shown in Figs 5 and 6.

Table 3 Structures and binding affinities of HupA analogues and the differences in interactions with AChE



A B C D

	R_1	R_2	R_3	R_4	Binding affinity	Differences with HupA	Ref.
(-)-HupA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		
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D2	[illegible]				[illegible]	[illegible]	[illegible]
D3	[illegible]				[illegible]	[illegible]	[illegible]

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the reason for the thus far unexplained slow binding reaction of the inhibitor[13].

In summary, the crystal structure of the HupA-TcAChE complex reveals an unexpected orientation of the ligand within the active site, as well as unusual protein–ligand interactions and a significant change in the main-chain conformation of the protein. This information should be of value for the design and analysis of analogues of HupA with improved pharmacological characteristics.

Methods

TcAChE was purified as described previously[17], with one modification: tetramethylammonium was used instead of the bisquaternary ligand, decamethonium, in the elution of the enzyme from the affinity column, to ensure the absence of the latter inhibitor from the active-site gorge of the purified enzyme[9].

Crystals of native TcAChE were obtained using the hanging-drop vapour diffusion method[14], employing as precipitant 35% polyethyleneglycol 200 in 0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 5.8, at 4 °C, and a protein concentration of 12 mg ml[-1]. For the HupA complex, crystals were soaked for nine days in a solution of ~10 mM optically pure HupA in crystallization mother liquor. X-ray diffraction data for both native and soaked crystals were collected at beam line X11 of the EMBL outstation at the DESY in Hamburg, Germany, on a 30 cm MAR Research image plate detector (λ=0.92 Å). The crystals were cooled to 0 °C using an Oxford Cryosystems cooling device. Both the native crystals and those of the complex diffracted to 2.2 Å but very weakly at high resolution. The data were processed using DENZO and SCALEPACK[15]. Details of data quality are given in Table 1.

The protein coordinates of the published native structure (PDB entry 1ACE)[16] were used for refinement, which consisted of several cycles of simulated annealing at 3000 K with harmonic constraints for the waters, followed by positional and individual B-factor refinement. All refinement was done using X-PLOR version 3.1[16], employing the Engh and Huber parameters[17]. All data between 8.0–2.5 Å resolution were used in the refinement, and maps were calculated using all data from 20.0–2.5 Å. Coordinates for the racemic mixture of HupA were obtained from the Cambridge Structural Database[18]; only the (−) stereoisomer gave a good fit to the observed electron density. The molecular geometry of the ligand was constrained during refinement. The density in the HupA complex for the two C-terminal residues, Ala 536 and Cys 537, is weak; the position of the sulphur atom (Cys 537 Sγ), which can be clearly seen, was fixed during initial refinement and released only in the final stage, and the occupancy for the two residues was set to 25%. In the native structure, no connecting density is seen for these two residues. The consistency of the protein geometry and of the positions of the water molecules was validated using ProCheck[38], Whatif[39] and OOPS[40]. Coordinates and structure factors for both the 2.5 Å native structure and the HupA complex have been deposited at the PDB, and have access codes 2ACE and 1VOT respectively.

For the analysis of the structure-function relationship of various HupA analogues, we generated a series of compounds using Insight[41], without energy minimization. The molecules so obtained were least-squares fitted to HupA in the crystal structure using the program O[31].

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ACUTE AND CHRONIC STUDIES WITH THE ANTICHOLINESTERASE HUPERZINE A: EFFECT ON CENTRAL NERVOUS SYSTEM CHOLINERGIC PARAMETERS

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Summary—High affinity choline transport, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) were assessed in rats after acute and chronic administration of the AChE inhibitor Huperzine A. Acute treatment: Forty-five min after a single injection of Huperzine A (0.5 mg/kg i.p.) the activity of AChE was significantly decreased by 15–30% in hippocampus, striatum and septum. The activity of ChAT was not altered. In the hippocampus high affinity choline transport was attenuated by 25%, whereas no effect in the striatum was observed. After 90 min, both inhibition of AChE and attenuation of high affinity choline transport had returned to control values. A dose of 0.1 mg/kg (i.p.) did not produce significant effects. Similar results were obtained with physostigmine (0.25 mg/kg), although the duration of inhibition of AChE was shorter than that with Huperzine A.

Chronic treatment: After 5 days (twice a day), at 0.5 mg/kg, the activity of AChE was significantly reduced by 20–30% in every region of the brain studied. High affinity choline transport in the hippocampus was reduced by 28%, 45 min after the last injection, but in the striatum there was no effect. The activity of ChAT was not affected in any region of the brain studied. Thus, acute or chronic treatment with Huperzine A: did not alter ChAT; reduced high affinity choline transport in the hippocampus in a transient manner; and had a longer duration of action as an AChE inhibitor than physostigmine. Moreover, tolerance to low-toxicity doses of Huperazine A was minimal, contrary to what has been observed with other inhibitors of AChE.

Key words—chronic, Huperzine A, anticholinesterase, HAChT, ChAT, Alzheimer's disease.

The new cholinesterase (AChE) inhibitor Huperzine A (Fig. 1) is an alkaloid extracted from a *Lycopodium* found in China. It was reported to ameliorate learning and memory retention in rodents (Lu, Shou and Tang, 1988; Tang, Han, Chen and Zhu, 1986; Zhu and Tang, 1988). Moreover, improvements in memory, lasting for several hours after a single intramuscular injection, were reported in patients affected by impairment of memory or Alzheimer's disease (AD) (Zhang, 1986).

Recently, the acute action of Huperzine A was investigated in the CNS of the rat by Tang, De Sarno, Sugaya and Giacobini (1989), who showed a sustained increase in levels of acetylcholine (ACh) in brain of several hours duration. At the doses used, the inhibition of cholinesterase lasted three times longer than with physostigmine as well as producing significantly fewer side effects than physostigmine or tetrahydroaminoacridine (THA) (Tang *et al.*, 1989).

However, the effect of Huperzine A on other central cholinergic parameters, such as the high affinity transport of choline and activity of choline acetyltransferase (ChAT) was not assessed *in vivo*. Neither was it determined if Huperzine A would be an effective cholinergic modulator during chronic treatment. Here, it is reported that the inhibitory action of Huperzine A on AChE *in vivo* was effective at smaller doses than previously reported and, moreover, it persisted after chronic treatment, in all areas of the brain. Huperzine A also produced a transient inhibition of the high affinity transport of choline in the hippocampus.

METHODS

Animals

Male Sprague–Dawley rats (Zivic Miller Laboratories, Allison Park, Pennsylvania) were used. At the time of the experiment, the rats weighted between 275 and 350 g. For the duration of the experiment, the rats were housed in groups of 2 on a 12-hr light–dark cycle. Food and water were available *ad libitum*.

Administration of Huperzine A

Huperzine A and the reference inhibitor of AChE physostigmine salicylate were solubilized in saline and injected intraperitoneally (i.p.). The chronic treatment consisted of 9 injections, over a period of 4 days (twice a day, hence). The 9th and last injection was administered 45 min prior to sacrifice.

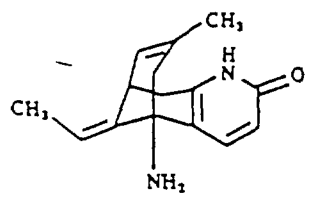

Fig. 1. Molecular representation of Huperzine A.

Dissection of tissue

After decapitation, the brain was quickly removed and the various regions of the brain studied were dissected from each hemisphere, on a chilled metallic plate, according to Glowinski and Iversen (1966).

Activity of ChAT

Dissected areas of the brain were homogenized in 19 volumes of sodium phosphate buffer (75 mM, pH 7.4, 4°C) and the homogenate was frozen at −70°C, until subsequent analysis of enzyme. After thawing, homogenate (10 μl, 6 mg protein per ml) was added in duplicate to 10 μl of buffer–substrate mixture (McCaman and Hunt, 1965; Spyker, Goldberg and Sparber, 1972) comprising: sodium phosphate, 75 mM (pH 7.4); NaCl, 600 mM; $MgCl_2$, 40 mM; physostigmine, 2.0 mM; bovine serum albumin, 0.05%; choline (Ch) iodide, 10 mM and [^{3}H]acetyl-coenzyme A, 0.87 mM. After 30 min of incubation at 37°C, the tubes were placed on ice and 150 μl of 3-heptanone, containing 75 mg/ml sodium tetraphenylboron, were added to each tube to extract the ACh (Fonnum, 1969). After vortexing, the samples were centrifuged and a 100 μl aliquot of the top (organic) layer was assayed for radioactivity, using liquid scintillation spectrometry.

Activity of acetylcholinesterase (AChE)

Dissected areas of the brain were homogenized in 19 volumes of sodium phosphate buffer (75 mM, pH 7.4, 4°C) and the homogenate was frozen at −70°C until subsequent analysis of enzyme. After thawing, the homogenate (10 μl, 6 mg protein per ml) was added in duplicate to 40 μl of buffer–substrate mixture, which contained: sodium phosphate (75 mM, pH 7.0, 4°C) and [^{3}H]ACh iodide (10 mM). After 20 min of incubation at 37°C, the tubes were placed on ice and 150 μl of sodium tetraphenylboron/3-heptanone were added to each tube to separate ACh from the acetate (Fonnum, 1969). The samples were vortexed, centrifuged and placed at −70°C, until the bottom (aqueous) layer was frozen; the top (organic) layer was then removed by aspiration. Subsequently, the aqueous layer was thawed and a 25 μl aliquot was assayed for radioactivity, using liquid scintillation spectrometry.

High affinity transport of choline

Dissected areas of the brain were homogenized in 19 volumes of sucrose (0.32 M, 4°C) and centrifuged (1000 g, 10 min, 4°C). The supernatant was then recentrifuged (20,000 g, 20 min, 4°C) and the resultant pellet was resuspended in 19 volumes of sucrose (0.32 M, 4°C). Duplicate aliquots (50 μl) of the suspension were then added to 500 μl of buffer (pH 7.4) comprising: Ch, 1.0 μM; [^{3}H]Ch, 0.28 μCi; NaCl, 126 mM; KCl, 9.6 mM; $MgSO_4$, 4.2 mM; $CaCl_2$, 2.4 mM; dextrose, 10.0 mM and Tris base, 40.0 mM. In Na^+-free buffer, 252 mM sucrose was substituted for sodium. After 8 min of incubation at 30°C, 3 ml of buffer (4°C) were added to each sample and tissue was collected onto GF/F filters (Whatman), by vacuum filtration. After washing with 10 ml of cold buffer, the filters were placed in scintillation vials and were assayed for radioactivity by liquid scintillation spectrometry. The Na^+-dependent high affinity transport of choline was defined as the amount of choline transported into tissue, in the presence of Na^+, minus that accumulated in the absence of Na^+ (Yamamura and Snyder, 1973). Protein was assayed according to Lowry, Rosebrough, Farr and Randall (1951).

Statistical analysis

Differences were compared by multiple analysis of variance and *post-hoc* analysis, using the SYSTAT Statistical System (Evanston, Illinois, U.S.A.).

RESULTS

Figure 2 illustrates the effects of a single injection of small doses of Huperzine A on the activity of AChE in various regions of the brain. The data indicate that the inhibition of esterase was dose- and time-dependent in hippocapus, striatum and septum. At 45 min after the injection, the dose of 0.1 mg/kg (i.p.) induced a slight but non-significant reduction in specific activity of AChE. At 0.5 mg/kg (i.p.), the activity of AChE was more strongly reduced ($P < 0.01$, < 0.001, < 0.005 in hippocampus, striatum and septum, respectively). At these small doses,

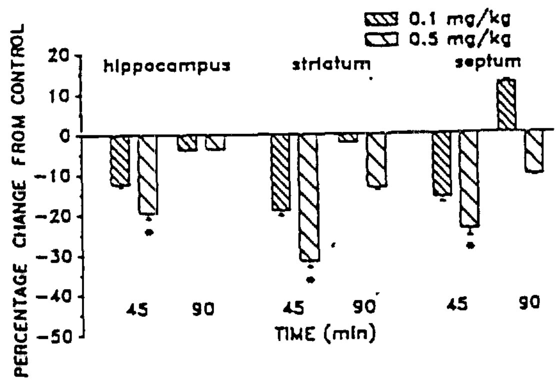

Fig. 2. Effect of acute injection of Huperzine A on activity of AChE in various regions of the brain. Values represent mean ± SEM. Two-way ANOVA (repeated measures), $P < 0.001$. Multiple contrasts analysis for dose-effect at 45 min: 0.1 mg/kg, $P = 0.52$; 0.5 mg/kg, $^*P < 0.01$. At 90 min: non-significant (N.S.). N = 4–8 rats/group.

the inhibition of AChE was mostly reversed by 90 min after the injection in all regions of the brain studied.

By comparison, a single injection of physostigmine (as the salicylate, 0.25 mg/kg i.p.) resulted in a more profound reduction in activity of AChE than that seen with Huperzine A (Fig. 2), ranging from 30 to 50% in parietal cortex, septum, hippocampus and striatum at 15 min after the injection (results not shown). However, the activity of AChE had reverted to control levels by 30 min after injection of physostigmine.

The specificity of Huperzine A on the metabolism of ACh was assessed by determining, in parallel, the activity of ChAT in each sample. The ACh-forming enzymatic activity was not influenced *in vivo* in the hippocampus or in the striatum by Huperzine A (results not shown). The specificity of Huperzine A on this cholinergic parameter, ChAT, was further compared to that of physostigmine (0.25 mg/kg i.p., 15 min after the injection) in cortex, septum, striatum and hippocampus. Physostigmine had essentially no effect on the activity of ChAT *in vivo* (results not shown).

As shown in Fig. 3, a single injection of Huperzine A produced a transient inhibition of the high affinity transport of choline in hippocampal synaptosomes. The transport activity was significantly ($P < 0.01$) reduced at 45 min, at the dose of 0.5 mg/kg (i.p.), whereas there was essentially no effect at 0.1 mg/kg (data not shown). By 90 min, the transport had returned to control values. High affinity transport of choline in the striatum was measured in parallel in the same animals at 45 and 90 min after the injection. The data in Fig. 3 show clearly that no inhibition of the uptake of choline took place at 0.5 mg/kg (i.p.), or at 0.1 mg/kg (i.p.) (data not shown).

The high affinity transport of choline was also assessed in various regions of the brain of rats injected with physostigmine (0.25 mg/kg i.p.). At

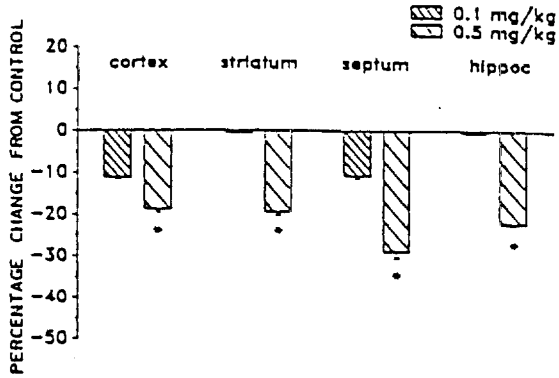

Fig. 4. Effect of 9 chronic injections of Huperzine A (4.5 days) on activity of AChE in various regions of the brain. Values represent mean ± SEM. Two-way ANOVA (repeated measures), $P < 0.001$. Multiple contrasts analysis for dose–effect: at 0.1 mg/kg, N.S.; 0.5 mg/kg, $^*P < 0.01$. $N = 6$ rats/group.

15 min, the transport was reduced significantly in hippocampus and parietal cortex by 34% and 37%, respectively, but not in the striatum (results not shown). By 30 min after the injection, the inhibition persisted significantly in the cortex and hippocampus.

The data in Figs 4 and 5 relate to the chronic treatment (twice a day for 4.5 days) with Huperzine A on the same parameters which were studied acutely. As shown in Fig. 4, the reduction in activity of AChE in the various regions of the brain, at the dose of 0.1 mg/kg (i.p.), did not reach significance. However, at 0.5 mg/kg, the results showed that activity of AChE was significantly reduced by 20–30%, in every region of the brain studied.

The high affinity transport of choline was similarly influenced by chronic treatment with Huperzine A, as is shown in Fig. 5. The slight reduction in transport of choline in the hippocampus was not significant at

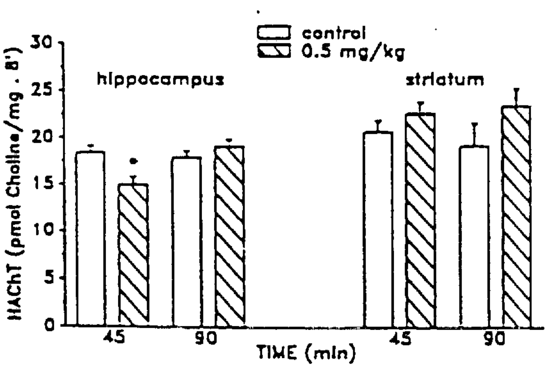

Fig. 3. Effect of acute injection of Huperzine A (0.5 mg/kg) on high affinity choline transport (HAChT) in hippocampus and striatum. Values represent mean ± SEM. Two-way ANOVA (hippocampus), $P < 0.005$. Single contrast analysis for dose–effect: at 45 min, $^*P < 0.01$; at 90 min, non-significant. Striatum: no significant differences. $N = 7$–10 rats/group.

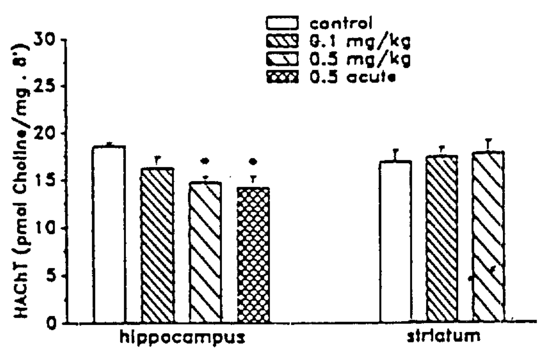

Fig. 5. Effect of 9 chronic injections of Huperzine A (4.5 days) on high affinity choline transport (HAChT) in hippocampus and striatum. Values represent mean ± SEM. One-way ANOVA (hippocampus), $P < 0.005$. Single contrast analysis for dose–effect: at 0.1 mg/kg: N.S.; at 0.5 mg/kg, $^*P < 0.01$. Striatum: no significant differences. The 0.5 mg/kg group (crosshatch) was injected acutely and used as control. $N = 5$–7 rats/group.

0.1 mg/kg. However, at 0.5 mg/kg, high affinity transport of choline was reduced by 28% ($P < 0.01$), 45 min after the last injection of Huperzine A, approximately to the same extent as in the acute controls included in this experiment. The high affinity transport of choline in striatal synaptosomes (Fig. 5), was not influenced by chronic treatment with Huperzine A, at either dose. The activity of ChAT was not affected *in vivo* in the hippocampus, striatum, cortex and septum after chronic treatment with Huperzine A (0.1 or 0.5 mg/kg i.p.) (results not shown).

Huperzine A-induced inhibition of the high affinity transport of choline was further investigated *in vitro*. Hippocampal synaptomes were incubated with Huperzine A, at concentrations ranging from 10^{-7} M to 10^{-4} M, for periods of 5, 15 and 45 min. No consistent effect of the inhibitor of cholinesterase on high affinity transport of choline could be detected *in vitro*.

DISCUSSION

From the present data it is clear that Huperzine A-induced inhibition of AChE activity was as potent after chronic, as it was after acute treatment. These results indicate that minimal tolerance to the drug occurred. This is important, since it is well established that tolerance develops to many of the effects of physostigmine (Costa, Schwab and Murphy, 1982; Genovese, Elsmore and King, 1988). It has also been shown that the response to various inhibitors of AChE varies considerably after a second injection (360 min), especially in the case of THA (Hallak and Giacobini, 1989).

In their recent study with Huperzine A, Tang *et al.* (1989) used doses of 2 mg/kg (i.m.), with maximum inhibition of AChE occurring at 60 min and reported side effects, such as fasciculations. Inhibition of AChE was also studied at 30 min using smaller doses (ranging from 0.1 to 2 mg/kg i.p.) and maximum inhibition of AChE with minimal side effects occurred between 0.50 and 1 mg/kg (i.p.) (Tang *et al.*, 1989). In the present study, using two small doses of Huperzine A, administered intraperitoneally, at 45 min, it was observed that inhibition of AChE was not very effective at 0.1 mg/kg (i.p.). However, although inhibition of AChE attained 30–50% with physostigmine (0.25 mg/kg i.p.), as compared to 15–25% with Huperzine A (0.5 mg/kg i.p.) in various regions of the brain, it was observed that the duration of inhibition of AChE was longer than that with physostigmine. These results agree with previous findings (Tang *et al.*, 1989). Furthermore, at the small dose of 0.5 mg/kg (i.p.), no mortality or any side effects were observed, even after chronic treatment.

The action of Huperzine A on the activity of ChAT was also investigated *in vivo*. Acute or chronic treatment with Huperzine A did not alter the activity of ChAT in any region of the brain studied. This finding complements the study of Hallak and Giacobini (1989), who reported no effect of various inhibitors of AChE *in vitro* (other than Huperzine A) on purified ChAT. Therefore, the reported *in vivo* increase in levels of ACh by Huperzine A (Tang *et al.*, 1989) was likely not to be mediated through an increase in the rate of synthesis of ACh.

In the same study, Tang and his coinvestigators showed that electrically-evoked release of ACh was not influenced by Huperzine A in slices of hippocampus. Neither was the release of ACh influenced by physostigmine, unless large concentrations were used (Hallak and Giacobini, 1989). Thus, it appears that release of ACh *in vivo* also may not be influenced by Huperzine A.

Another important effector of metabolism of ACh is the high affinity transport of choline (Tuček, 1985). According to the present studies, acute or chronic administration of Huperzine A was a potent inhibitor of high affinity transport of choline in the hippocampus *in vivo*. Physostigmine (Atweh, Simon and Kuhar, 1975; Sherman and Messamore, 1988) and THA (Sherman and Messamore, 1988) were also found to have a similar effect on transport of choline *in vivo*. However, in those studies, large doses of inhibitors of AChE, often accompanied by toxic effects, were used. Atweh *et al.* (1975) clearly showed that drugs affecting the turnover of ACh *in vivo* influenced the high affinity transport of choline, accordingly. For instance, physostigmine was shown to reduce turnover of ACh (Saelens, Simke, Schuman and Allen, 1974; Trabucchi, Cheney, Hanin and Costa, 1975) and muscarinic agonists, which increase turnover of ACh, increased high affinity transport of choline (Atweh *et al.*, 1975). The effect of inhibition of AChE on uptake of choline is believed to be mediated through a regulatory pre-synaptic control of high affinity transport of choline in response to the increase in content of ACh following inhibition of esterase (Yamamura and Snyder, 1973; Jope, 1979; Tamaru and Roberts, 1988; Breer and Knipper, 1990). The present results support this contention, since the effect of Huperzine A was completely reversible with time (Fig. 2) and not mediated through a direct interaction with the transporter (results not shown). Physostigmine also did not show any direct effect *in vitro* on synaptosomes in brain (Yamamura and Snyder, 1973), contrary to neostigmine (Yamamura and Snyder, 1973; Simon, Mittag, and Kuhar, 1975). These results indicate that inhibition of AChE may influence the high affinity transport of choline through a feedback-type regulation, rather than by operating directly on the transporter.

Hallak and Giacobini (1987) have hypothesized that *in vivo* treatment with an inhibitor of AChE "which would not decrease turnover of ACh, would maintain long-lasting levels of the neurotransmitter in the brain". Such may indeed be the case with Huperzine A. Although the present results could be interpeted as an indication that Huperzine A operates in the CNS according to the same mechanisms as those postulated for physostigmine, only the specific determination of the turnover of ACh will resolve the issue.

Another finding of this study that remains to be addressed is why the high affinity transport of choline was not decreased in the striatum, despite a potent reduction in the activity of AChE by both Huperzine A and physostigmine in this region of the brain. The striatum contains the greatest concentration of ACh in the brain (Sethy, Roth, Kuhar and Van Woert, 1973). Nevertheless, inhibition of AChE may not be accompanied by a significant elevation of ACh in striatum (Tang *et al.*, 1989). De Sarno, Pomponi, Giacobini, Tang and Williams (1989) have also shown that, after injection of a long-lasting derivative of physostigmine, increases in levels of ACh showed marked regional differences. Moreover, it has been appreciated for some time that regional variations exist among the effects of drugs on the high affinity transport of choline (Jope, 1979) and that the striatum often differs from other areas of the brain in its cholinergic responses to pharmacological challenges (Wecker and Dettbarn, 1979; Sherman, Zigmond and Hanin, 1978).

In conclusion, it has been demonstrated that low-toxicity doses of Huperzine A could be used for several consecutive days and still exhibit full potency; hence, tolerance to Huperzine A, if it occurred, was minimal. Furthermore, the differences that have been shown in inhibition of AChE induced by Huperzine A and physostigmine are further indications that Huperzine A may be more effective and less toxic than physostigmine when a long term inhibition of AChE is required, e.g. in clinical treatment of diseases manifesting a cholinergic hypofunction.

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